

THE SINGAPORE EXPERIENCE IN  
HUMAN IN VITRO FERTILIZATION AND EMBRYO REPLACEMENT:  
ENDOCRINOLOGICAL CONTRIBUTIONS IN THE  
FOLLICULAR AND LUTEAL PHASES.

A thesis submitted for

Doctor of Medicine,

National University of Singapore.



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M.B., B.S.; M.Med (O&G); M.R.C.O.G.

March 1987.

This thesis and its related work

is dedicated to

my wife

Kiat Piah

and

my son

Yifan



Timing is the name of the game; a day too late or too soon and the endometrium is not receptive. A little before or a little beyond the blastocyst stage and the embryo cannot implant. Each event depends on the others, the whole orchestrated by the signals that flicker to and fro between embryo and mother.

Klopper A. Commentary: The first days.

British Journal of Obstetrics and  
Gynaecology.

1983;90:593-594

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## ABBREVIATIONS

IVF	:	In vitro fertilization
ER/ET:		Embryo replacement/transfer
GnRH	:	Gonadotropin releasing hormone
FSH	:	Follicle-stimulating hormone
LH	:	Luteinizing hormone
PRL	:	Prolactin
E <sub>2</sub>	:	Estradiol
P	:	Progesterone
hCG	:	Human chorionic gonadotropin
hMG	:	Human menopausal gonadotropin
i.u.	:	International units
Fig	:	Figure
g.	:	gramme
mg.	:	milligramme
ug.	:	microgramme
ng.	:	nanogramme
pg.	:	picogramme
ml.	:	millilitre

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## SUMMARY

This thesis is an investigation of the endocrinological events in patients undergoing in-vitro fertilization (IVF) and is based on the IVF program that began in 1982 in Kandang Kerbau Hospital. The first 22 patients were either stimulated with clomiphene alone or were unstimulated (phase I : July 1982 - May 1983); clomiphene citrate 150 mg/day for 5 days from day 2. Ultrasound scans were done daily from day 8. The subsequent 105 cycles were stimulated with a combination of clomiphene and hMG (phase II : September 1983 to September 1985). Clomiphene 100 mg/day was given from day 2 for 5 days. On day 6, hMG injections were given; regime I was 1 ampoule daily from day 6 to 8, while regime II was 2 ampoules daily for the same duration. From day 9, the hMG dosage was dependent on the plasma  $E_2$ . An ultrasound scan was done only on day 9. In both phases, urine was collected regularly after admission and LH values determined with Hi-Gonavis twice daily. In the absence of the LH surge, hCG was given based on  $E_2$  or ultrasound parameters. Laparoscopy was done 24-26 hours from the onset of the LH surge, or 34-36 hours from the hCG injection. In phase I, the follicular mean plasma  $E_2$  level varied from 195 pg/ml/follicle 7 days before laparoscopy to 633 pg/ml/follicle on the day of the laparoscopy in the clomiphene-treated patients, and from 87 pg/ml/follicle 7 days before laparoscopy to 473

pg/ml/follicle on the day of the laparoscopy in the unstimulated group. In phase II, there was no difference in the  $E_2$  response up to the day of laparoscopic egg recovery for the two regimes (mean of 589.9 pg/ml/follicle and 661.8 pg/ml/follicle for regimes I and II respectively). Spontaneous LH surge was observed in 4 of 9 cases in regime I, and 6 of 10 cases in regime II. When hCG was given earlier at  $E_2$  of 500 pg/ml/follicle, there were significantly ( $p < 0.02$ ) more pre-ovulatory oocytes (98.9%), compared with the group when hCG was given later (92.4%). However, fertilization and cleavage was significantly less ( $p < 0.05$ ) for the earlier group (46.2% and 63.9% respectively). When the Hi-Gonavis assay was checked with RIA, the false negative rate was 7.4%, while the false positive rate was 16.7%. In the false positive group, 65% of the eggs were mature, and these fertilised and cleaved. On the other hand, in the true positive group only 36% of the eggs were mature, and only 58% fertilised and cleaved. Daily ultrasound in phase I showed a significant correlation in follicular growth in clomiphene-induced patients with 1 and 2 follicles ( $p < 0.001$ ), but there was no such correlation in unstimulated patients. Though there was no difference in  $E_2$  levels in the luteal phase, there was a significantly higher plasma P in the progesterone supported group with spontaneous LH surge on days 10, 12 and 14.



## CHAPTER 1:

### INTRODUCTION

This thesis is an investigation of the endocrinological events in patients undergoing in-vitro fertilization (IVF). Such events are important because it forms the basis of management of the patients, leading to egg recoveries and finally pregnancies. The most critical is the quality of the egg, and, now increasingly so, the quality of the sperm. The egg is nurtured over 10 to 14 days of dynamic endocrine changes. Such changes can be followed from studies in peripheral blood, mainly designed to give a better understanding of natural cycles. Since the use of ovulatory drugs (Gemzell, Diczfalusy, Tillinger, 1958; Kistner, 1966) there is a better understanding of stimulated cycles. In IVF, initially there was a debate of unstimulated versus stimulated cycles (see Trounson, Leeton, Wood et al, 1981), but it is now recognised that stimulated cycles give better pregnancy rates, mainly because of increased numbers of oocytes and consequently embryos replaced (Gronow, Martin, McBain et al, 1985). This in turn has lead to overstimulation (Jones 1984) to increase the number of eggs collected. However, not much is known of controlled overstimulation, which is different from hyperstimulation (Rabau,

David, Serr et al, 1967). The purpose of this thesis is to try to shed some light in this area of ovarian stimulation in an IVF program.

This thesis is based on the IVF program in the Department of Obstetrics & Gynaecology, National University of Singapore. An IVF program to help childless couples was started by the Department of Obstetrics & Gynaecology in 1982 at the Kandang Kerbau Hospital, Hampshire Road. When the Department moved to the National University Hospital in Kent Ridge in October 1985, the IVF program was then based in the newly formed Division of Reproductive Endocrinology.

Over the years since the commencement of the IVF program, various superovulation regimes were used. At the Kandang Kerbau Hospital, the first 22 patients were either superovulated with clomiphene alone or were unstimulated. For convenience in presentation, this period shall be designated phase I in this study (July 1982 - May 1983). The subsequent 100 patients (September 1983 to September 1985) were superovulated with a combination of clomiphene and human menopausal gonadotropin (hMG) and this is represented as phase II.

When the IVF program became based in the National University



Hospital (phase III), the superovulation regime was changed to a combination of hMG with purified follicle-stimulating hormone (FSH). This regime was also used for the gamete intra-fallopian transfer (GIFT) method of treatment of infertility which was also available to patients as part of the Department's comprehensive subfertility treatment program.

The objective of this study was to evaluate the endocrinological data collected from the program while it was based at Kandang Kerbau Hospital (phases I & II) during the period 1982 - 1985.

The thesis is presented in the following manner:

1. Literature review.

A review of the endocrine manipulation which has contributed to the superovulation normally practiced for IVF.

2. Materials and methodology.

This includes an analysis of patients, their indications, description of their stimulation, the egg recovery procedure, a brief description of the fertilization and growth in vitro of the embryos, the embryo replacement, and finally the luteal phase management.

3. Singapore's first test-tube baby.

A case presentation of our first success.

4. Follicular phases of unstimulated and clomiphene-induced cycles.

Comparison of  $E_2$  response in phase I cycles where the patients were either unstimulated or stimulated with clomiphene.

5. Follicular phase of clomiphene-hMG stimulated cycles.

Comparison of  $E_2$  response in two clomiphene-hMG regimes.

6. Earlier administration of hCG.

The effect of earlier administration of hCG on oocyte quality.

7. Spontaneous LH surges.

The use of Higonavis for detection of spontaneous LH surge.

8. The combination of ultrasound with  $E_2$  estimation to predict spontaneous LH surge.

The effect of diminished ultrasound scanning frequency and daily  $E_2$  estimation on the ability to predict spontaneous LH surges.

9. Progesterone support in luteal phase.

A randomised trial of luteal phase support with P.

10. Conclusion.

11. References.

CHAPTER 2

LITERATURE REVIEW

- a) Endocrine relationships in normal ovulatory cycles.
- b) Induction of ovulation.
- c) Overstimulation for in-vitro fertilization.
- d) Possible complications in overstimulation.

SYNOPSIS

Induction of ovulation is important in the treatment of female subfertility. Recent understanding in the neurophysiology of ovulation control has assisted in the therapy of anovulatory women. More recently, augmentation of ovulation is employed in ovulatory women in order to overstimulate them for the purpose of collection of extra eggs, especially in IVF. Drugs used for this purpose include clomiphene, gonadotropins and GnRH; most of them are used in combination. This literature review surveys the field of ovulatory control and its use for IVF.



## INTRODUCTION

Induction of ovulation is one of the major advances in the past few decades in the treatment of anovulatory infertility. There are 4 major indications for ovulation induction: 1) fertility in anovulatory infertile women; 2) timing of ovulation in irregularly ovulating women who may also need insemination with donor semen; 3) increase in oocyte numbers and control of maturation for IVF and gamete intra-fallopian transfer (GIFT); or 4) as a test for hypothalamo-pituitary-ovarian axis function.

This survey shall be limited to the more recent development of augmentation of ovulation in ovulatory women in order to over-stimulate them for the purpose of collection of extra eggs. Drugs used for this purpose include clomiphene, gonadotropins and GnRH; most of them are used in combination. These approach is used principally in the newer reproductive technologies, especially IVF, GIFT, and related areas such as freezing of embryos.

## ENDOCRINE INTERRELATIONSHIPS IN THE MENSTRUAL CYCLE.

The hypothalamo-pituitary-ovarian (HPO) axis is the pathway in which the 3 essential organs in ovulation send their messages

(hormones) from one to another. This axis is finely-tuned and can be easily disturbed. The main hormones involved are from the hypothalamus (GnRH); the pituitary (gonadotropins - FSH and LH; PRL); and the ovary (steroids -  $E_2$  and P, and the protein - inhibin).

GnRH is a decapeptide (Matsu, Baba, Nair et al, 1971) secreted by the nerve bodies found mainly in the supraoptic and prechiasmatic regions of the hypothalamus. Their axons descend into 1) lateral palisade zone of median eminence (majority), and 2) medial basal hypothalamus and mamillary area. The self-priming effects of GnRH comprise of GnRH pulses every 90 minutes in the follicular phase (Knobil and Wildt, 1982). Each pulse releases gonadotropins and increases their synthesis. The pattern of GnRH secretion, and not the amount of decapeptide, is of critical importance (Knobil and Wildt, 1982).

GnRH secretion is also influenced by estrogens. There are 2 types of such "feedback":

- 1) Negative feedback - ovariectomy results in a rise in FSH and LH; oestrogens given to postmenopausal women result in lowering of FSH and LH levels.



2) Positive feedback which triggers neural and pituitary events that accompany the pre-ovulatory surge of gonadotropin. This surge is due mainly to increased pituitary response, as well as to increased hypothalamic secretions of GnRH. Hence, the positive feedback effects of  $E_2$  are exerted mainly at the pituitary gland; hypothalamic GnRH secretion plays an obligatory but permissive role (Knobil and Wildt, 1982).

The neurotransmitters of importance are dopamine (DA) and norepinephrine (NE). DA inhibits while NE facilitates GnRH secretion (McCann and Moss, 1975).. Less importantly, acetylcholine and gamma-aminobutyric acid (GABA) stimulate, while serotonin inhibits. Elevated PRL stimulates DA secretion (Gudelsky, Simpkins, Mueller et al, 1976), thus inhibiting GnRH release.

Gonadotropin secretion is pulsatile, reflecting its pulsatile control from the hypothalamus (Yen, Tsai, Naftolin et al, 1972). The number of FSH and LH pulses increases during the first few days after menstruation, and remains at about once every 90 minutes until after ovulation (Wildt, Schwilden, Wesner et al, 1983). No significant change in pulse frequency occurs during this time, including the midcycle surge (Wildt, Schwilden, Wesner et al, 1983), thus supporting the view that the dramatic increase

in gonadotropin secretion is not dependent upon changes in the pattern of hypothalamic GnRH secretion (Knobil, 1980). However, as the follicular phase progresses, the amplitude of LH pulses decreases; moreover, the FSH/LH ratio decreases as FSH decreases from 12-20 mIU/ml in the beginning of the cycle to 4-8 mIU/ml at mid-cycle while LH values remain between 8-12 mIU/ml (Wildt, Schwilden, Wesner et al, 1983). In the luteal phase, there are dramatic changes in LH pulse frequency; there is a reduction in LH pulse frequency, but with considerably larger amplitudes (except for the LH surge). With the onset of menstruation, pulse frequency increases dramatically and this continues for the first few days of the follicular phase until a pulse frequency of about once every 90 minutes is reached (Wildt, Schwilden, Wesner et al, 1983).

When gonadotropin levels are taken once daily through the menstrual cycle, they have a characteristic pattern. FSH increases just before menses of the preceding cycle, due to decreasing  $E_2$  levels. FSH rises up to 4 to 5 days into the follicular phase, stimulating the primary pool of follicles. However, small amounts of LH seems to greatly enhance this stimulatory effect of FSH, and seems to be necessary for steroidogenesis (Jones, Ruehsen, Johanson et al, 1969; Midgley, Niswender, Gay et al, 1971; Bertrand, Coleman, Crooke et al,



1972). As the follicles become activated, they secrete E<sub>2</sub>. Follicular growth increases rapidly during the second week. As the E<sub>2</sub> levels increase, there is a negative feedback onto the pituitary and hypothalamus which results in decreasing levels of FSH. The interaction between estrogen and FSH may be important in the selection of the dominant follicle (Fritz and Speroff, 1983). The "ovulatory quota" of one egg can only be overridden with exogenous gonadotropin. The fall in FSH removes the support for other less developed follicles, which then undergo atresia. LH levels rise slowly during this period. When E<sub>2</sub> reaches a critical value, it activates a positive feedback, which results in an LH surge. This surge lasts for 3 days, with the peak on the second day. The LH surge initiates luteinisation and causes ovulation by a mechanism presently unknown.

Steroid production is dependent on the enzymes present in the preantral follicle, and the granulosa cells at this stage are able to synthesize all three classes of steroids, though in limited quantities (McNatty, Makris, De Grazia et al, 1979). However, substantially more estrogens are produced than androgens or progestins, through the aromatisation of androgens. This aromatisation is induced through FSH, which also results in an increase in FSH receptors. Neither FSH or LH is usually detected in antral fluid unless their blood levels are elevated. LH is not

normally present in the follicular fluid until or just after the LH surge. If LH levels are prematurely elevated in plasma or follicular fluid, reductase activity is stimulated and there is elevated androgen levels resulting in decrease in mitotic activity of granulosa cells and degeneration. The thecal cells are also important as they produce androgens in response to LH. The androgens then diffuse into the granulosa cells and are aromatised to oestrogens through FSH-induction. This is known as the two-cell, two-gonadotropin concept of ovarian steroidogenesis (Dorrington and Armstrong, 1979). The follicular fluid concentration of E2 (absolute and relative to androgen) increases in proportion to the activity of the granulosa cell aromatase system and is highest in the preovulatory follicle.

Although many follicles undergo a certain amount of growth and development during the reproductive cycle, only a certain number eventually reaches the stage of ovulation, while the rest become atretic. Several concepts have been proposed to explain the process of atresia (Ryan, 1981). Even though all follicles are exposed to the same extraovarian regulators, eg FSH and LH, there are considerable differences in the micro-environment of individual follicles (Van Look and Baird, 1980; McNatty, Smith, Makris et al, 1979; Bomsel-Helmreich, 1983). Atresia is thought to be due to differences in aromatizing capacity, and may be



caused by different sensitivities of follicles towards gonadotropins. It has been suggested that none of the follicles present in the beginning of a menstrual cycle which has the potential to reach the pre-ovulatory stage will attain full maturation unless it has been exposed sufficiently to the action of FSH to pass a critical threshold (Hillier, Van Hall, van den Boogard et al, 1891). This "threshold" is apparently determined for each follicle by one or more variables such as (a) local vascularization optimizing the accessibility of follicles to gonadotropins (Zelevnik, 1981), (b) the number of granulosa cells and/or gonadotropin receptors per cell which would determine a differential response to gonadotropins (McNatty, 1981), and (c) interfollicular levels of substances which modulate the actions of gonadotropins per se (Hillier, Van Hall, van den Boogard et al, 1891; Zelevnik, 1981).

In addition to steroids, gonadotropins and GnRH-like intra-ovarian substances, there are other non-steroidal factors present in the follicle which influence follicular development (Daume, Chari and Hillensjoe, 1984). These include inhibin/folliculostatin, oocyte maturation inhibitor (OMI), follicle-stimulating hormone-binding inhibitor (FSH-BI), luteinization inhibitor (LI), and luteinization stimulator (LS).

Inhibin is a protein hormone believed to suppress FSH release from gonadotrophs (De Jong and Sharpe, 1976). Its secretion decreases as ovulation approaches; hence the removal of its inhibitory activity allows the mid-cycle surge of FSH (Kimura, Katoh, Taya et al, 1983). There are 2 molecular species with inhibin activity in human follicular fluid, a large inhibin with a molecular weight of 23,000 daltons, and a small inhibin with a molecular weight of < 1000 daltons (Chari, Hopkinson, Daume et al, 1979). The large inhibin has been shown to inhibit GnRH secretion by the hypothalamus (Chari, Duraiswami, Daume et al, 1981). Inhibin has also been implicated as a regulator of interovarian function, with exogenous inhibin resulting in induction of follicular atresia (Daume, Chari and Hillensjoe, 1984). In addition to suppression of FSH release, small inhibin is also capable of inhibiting secretion of P by rat and human granulosa cells as well as the maturation of rat oocytes (Hillensjoe, Chari, Magnusson et al, 1981).

The oocyte maturation inhibitor (OMI) was assumed because the oocyte, under physiological conditions, does not undergo the maturation division before ovulation (Channing, Anderson, Hoover et al, 1982). OMI from porcine follicular fluid has been partially purified, and appears to be a peptide with a molecular weight of less than 2000 (Tsafriri, Pomerantz and Channing,



1976).

The follicle-stimulating hormone binding inhibitor (FSH-BI) was first described by Darga and Reichert (1978) which, at the appropriate site is capable of inhibiting the binding of its hormone to its receptor, thus regulating ovarian function. It is a small molecule, about 1200 daltons. It is also not unique to follicular fluid, having been described from serum and ascitic fluid (Reichert, Sanzo, Darga, 1979).

As the follicle matures it gains a substance that enhances luteinization (luteinizing stimulator, LS). Its stimulatory actions on granulosa cells include morphological maturation, progesterone and estrogen secretion, enhanced responsiveness to gonadotropins and activation of ornithin decarboxylase (Daume, Chari and Hillensjoe, 1984).

On the other hand, there is in fluid from small follicles a factor that inhibits spontaneous luteinization of granulosa cells, P secretion, prostaglandin  $F_2$  secretion, and responsiveness to LH and FSH (Daume, Chari and Hillensjoe, 1984). This factor is called the luteinization inhibitor (LI).

Receptors for FSH are present on granulosa cells and not thecal

cells. Their numbers increase with follicular growth through cell division for the number of receptors per cell remains constant. FSH stimulates formation of its own receptors, as well as LH receptors; it also increases cAMP levels and enhances ovarian steroidogenesis (Edwards, 1980). LH receptors appear late. In the preovulatory follicle, the LH surge results in a co-ordinated series of changes (Edwards, 1980). The endocrine changes that occur include decline in receptors and binding of LH, decrease in the production of thecal androgens, reduction in aromatization by granulosa cells, and stimulation of P synthesis by granulosa cells. Preovulatory changes induced by the LH surge also include synthesis of prostaglandins, glycosaminoglycans and proteases as well as resumption of meiosis in oocytes. While the above changes are ascribed to LH, FSH stimulates granulosa cells to synthesize plasminogen and mucin. Follicles become desensitised to LH a few hours after the surge.

The mechanism of ovulation is obscure. Both FSH and LH stimulate prostaglandin synthesis by granulosa cells (Tsafriri, Lindner, Zor, et al, 1972). Furthermore, both gonadotropins and prostaglandins stimulate granulosa cells to secrete an activator that converts plasma-derived follicular plasminogen into plasmin, a protease that may be important in the dissolution of the lamina basalis during ovulation (Espey, 1974). The functional sig-



nificance of ovarian contractility is uncertain, and it may not be required for ovulation (Espey, 1978). Increased intrafollicular pressure in precipitating follicular rupture is now thought to be unimportant.

E<sub>2</sub> falls 24 hrs before ovulation and is thought to be due to activation of the delta-4 pathway in the granulosa cells by the LH surge from the delta-5 pathway in the thecal cells (Edwards, 1980); E<sub>2</sub> then rises with P produced by the corpus luteum. The pre-ovulatory fall in E<sub>2</sub> coincides with an increase in sensitivity of the follicle to LH at mid-cycle (Zelevnik, Midgley and Reichert, 1974; Brown, 1978). LH is necessary for corpus luteum function (Van de Wiele, Bogumil, Dyrenfurth, et al, 1970). There are LH receptors in human corpora luteal tissue (Lee, Coulam, Jiang et al, 1973).

and conceive on placebo alone (Evans, 1975).

### 1. Clomiphene citrate and other anti-estrogens

Clomiphene citrate belongs to a group of steroid-like compounds called stilbenes, along with cyclofenil and tamoxifen, with ovulatory function (Taubert and Kuhl, 1986). The discovery of a nonsteroidal analogue of  $E_2$ , clomiphene citrate, that has a stimulatory effect on ovarian function was made by Kistner and Smith (1960), and Greenblatt and co-workers (Greenblatt, Barfield, Jungck et al, 1961). Since then, treatment with clomiphene has probably helped more infertile women to conceive than any other infertility treatment.

Clomiphene (as with cyclofenil and tamoxifen) are nonsteroidal agents that have a structural similarity to the potent estrogen diethylstilbestrol. Although these compounds are not steroids but triphenylethylenes, their steric configuration shows a remarkable structural similarity to  $E_2$ , and therefore enables binding to estrogen receptors (Taubert and Kuhl, 1986). The binding activity of clomiphene citrate to uterine estrogen receptors has been reported to be lower than that of  $E_2$  (Ruh and Ruh, 1974). Clomiphene citrate has not been found to exert any progesta-



tional, androgenic or anti-androgenic biological activity (Owman and Hafez, 1980).

$E_2$  activity is related to binding of its cytoplasmic receptor. This results in an allosteric change of its receptor configuration ("activation"), leading to dimerization of the estrogen receptor complex (Little, Szendro, Teran et al, 1975). This complex is then rapidly translocated to the nucleus where it acts preferentially with a small number of specific binding sites on the genome, along with nonspecific interactions of the receptor protein with DNA (Yamamoto and Alberts, 1974). The only limiting factor is the amount of receptor available (Katzenellenbogen, Bhakoo, Ferguson et al, 1979). The gradual movement of the steroid receptor complex to the nucleus, and the maintenance of elevated levels of nuclear receptors for a longer period of time involves the depletion of cytoplasmic receptors for some hours (Chan and O'Malley, 1976). During this period the organ is insensitive to additional hormone. Replenishment of the receptor (by de novo synthesis as well as recycling after dissociation from the nuclear sites) results in return of sensitivity. However, the longer an estrogen dissociates from the receptor complex, the longer is the duration of nuclear retention, and the greater the biological effect (Weichmann and Notides, 1980). The full sequence of estrogen response of a target cell requires a minimal



number of estrogen receptor complexes to be retained in the nucleus for several hours.

The estrogen antagonist, in contrast to  $E_2$ , is incapable of inducing the adequate allosteric configuration of the receptor complex, and therefore has a different nuclear interaction. However, the earlier events are normal, ie. it is complexed with the cytoplasmic estrogen receptor and is translocated to the nucleus. More importantly, anti-estrogen action results in the failure of replenishment of the depleted cytoplasmic receptor. Moreover, their dissociation from chromatin is impaired, and they remain in the nucleus for a longer period of time, blocking the recycling of receptors back to the cytoplasm (Katzenellenbogen, Bhakoo, Ferguson et al, 1979; Chan and O'Malley, 1976). Clomiphene citrate has been found to persist in body tissues up to 3 months (Trounson and Leeton, 1982). Therefore, clomiphene is an antagonist not because it competes for cytoplasmic receptors, but because it maintains the depletion of cytoplasmic receptors, causing to a certain extent a state of estrogen-insensitivity of the target cell.

The manner in which ovulation is induced by clomiphene is still uncertain, although it is very likely to be due to a reduction in the negative feedback of endogenous estrogens. The augmentation

of gonadotropin secretion has not been localized specifically to either a hypothalamic or pituitary event. It has been suggested that clomiphene citrate depletes estrogen receptors in the hypothalamus (Etgen, 1979; Kahwanago, Heinrichs and Herrmann, 1970), stimulating GnRH release and a subsequent rise in LH and FSH (Miyake, Aono, Minnagawa et al, 1980). Alternatively, or concomitantly, clomiphene citrate may decrease the cytosol estrogen receptor content of pituitary cells, allowing an interruption of tonic gonadotropin suppression by estrogen (Etgen, 1979; Kahwanago, Heinrichs and Herrmann, 1970).

When clomiphene is administered in the early follicular phase, an increase of LH, and to a much lesser degree of FSH, is seen (Vandenberg and Yen, 1973); this is suggestive of a decrease in the feedback inhibition of the hypothalamic-pituitary axis by  $E_2$ . This positive effect on LH is enhanced by administration of clomiphene during the late follicular phase, but it has no effect on gonadotropin secretion during the luteal phase (Vandenberg and Yen, 1973).

The FSH elevation in response to clomiphene is thought to allow follicles other than the dominant follicle to become pre-ovulatory by increasing the amount of FSH available to them. This results in a rapid stimulation of  $E_2$  production, and continues to



increase markedly after cessation of clomiphene ingestion until a maximum is reached the day before the pre-ovulatory LH surge (Vandenberg and Yen, 1973). However, this continuous increase in  $E_2$  production can occur independently of serum gonadotropin levels, suggesting a direct effect of anti-estrogens on the ovary (Groom and Griffiths, 1976). Adashi (1984) proposed that the pro-fertility effect of clomiphene is due to the sum of direct effects on the hypothalamus, pituitary and ovaries.

Besides the long-loop feedback regulation of the HPO axis, an internal short-loop feedback control within the follicles appears to be responsible for the individual hormonal "micro-climate" of the follicular fluid (Taubert and Kuhl, 1981). This is supported by in-vitro culture experiments on granulosa cells; Zhuang, Adashi and Hsueh (1981) have reported that clomiphene augmented the gonadotropin-stimulated estrogen production in cultured granulosa cells. This direct positive feedback of clomiphene could be responsible for the four-fold increase in estrogen concentration in follicular fluid during clomiphene treatment as compared to normal ovulatory cycles (Smith and Kistner, 1963). The marked rise in intra-follicular  $E_2$  level leads to a relative autonomy from gonadotropins of the maturing follicle until ovulation. This could account for clomiphene-induced overstimulation of the ovary (Taubert and Dericks-Tan, 1976). On the other hand,



the direct action of clomiphene on the ovary can only take place when follicular development has reached a certain stage of maturation; this is seen clinically when clomiphene-induced ovulation can only occur in normoestrogenic women.

Clomiphene citrate is the first line treatment for amenorrhoeic and oligomenorrhoeic patients with normal FSH, LH and PRL levels. There are 2 stereo-isomers. The cis-form is an anti-estrogen and can produce ovulation in up to 78% of patients studied, while the trans-form is mildly estrogenic with an ovulation rate of 51% (Asch and Greenblatt, 1976). However, the preparation used clinically contains both the cis- and trans-form, and induces ovulation between 40 and 70%. Clomiphene acts on the hypothalamus and causes a rise in FSH and LH during its administration. This stimulates follicular growth and a pre-ovulatory peak occurs 3-9 days after completion of therapy (Jacobson, Marshall, Ross et al, 1968). However, probably because of different sensitivities of the hypothalamic receptors to the positive and negative feedbacks of estrogens produced by the ovaries, ovulatory responses differ and some patients remain anovulatory.

Treatment regimes with clomiphene vary with centres. The most common is with clomiphene from day 5 following for 5 days (Huppert, 1979). An alternative is from day 2 for 5 days. Some

centres prefer to go by the patients' cycle length. Dose starts from 50 mg daily and can be increased to 200 mg daily in 50 mg increments per cycle if anovulatory. Some patients may experience hot flushes at the higher dosages.

Response with clomiphene is more likely in less severe forms of hypothalamic dysfunction. In a series of 283 patients, Brown and co-workers (Brown, Pepperell and Evan, 1980) reported 85.3% of patients with anovulatory cycles ovulated, 74.0% of the oligomenorrhoeics, 56.3% of the patients with secondary amenorrhoea ovulated, and none of the patients with primary amenorrhoea ovulated. In the 3 groups that responded, the pregnancy rates are about half the ovulation rates. This low pregnancy rate has been ascribed to the anti-estrogenic effect of clomiphene on the cervical mucus.

In an analysis of 1034 pregnancies after clomiphene therapy in Japan, Kurachi and co-workers (Kurachi, Aono, Minagawa et al, 1983) reported a 2.3% visible malformations in the live-births. Abortion rate was 14.2% and stillbirth rate was 1.6%. There was also no correlation between the clomiphene dosage and malformation rate. Their conclusion was that clomiphene citrate does not give rise to an increased incidence of malformations.



Side-effects of clomiphene therapy include hot flushes (10.7%) and pelvic discomfort (7.4%). A small number of patients complain of visual disturbances and other vague symptoms.

Complications of clomiphene therapy are related to ovarian hyperstimulation and consist of enlargement of the ovaries and multiple pregnancies. Multiple pregnancy can be expected in 10%. Although the majority are twins (99%), triplets, quadruplets, quintuplets and even sextuplets have been reported.

## 2. Gonadotropins

The pituitary gonadotropins FSH and LH, the placental gonadotropin hCG and thyroid stimulating hormone (TSH) are closely related in that they are glycoproteins made up of 2 dissimilar subunits designated alpha and beta. The 2 subunits are linked by hydrophobic (noncovalent) binding and can be dissociated by treatment with 10 M urea at pH 4.5 or 1 M propionic acid and highly purified. The pure subunits are practically without biological activity but the activity is regenerated by allowing the 2 subunits to recombine.

Human FSH, hLH, hCG and hTSH have a common alpha subunit which



contains 92 amino-acids and two carbohydrate moieties. The beta subunits are different and provide the unique biological properties of the hormone. Nevertheless, portions of the beta polypeptide chain of one gonadotropin may be found in others. This is seen particularly in hLH and hCG where considerable homology in the polypeptide chain occurs, and these 2 gonadotropins probably share common receptor sites. Some of the properties of the subunits of the gonadotropins are shown below:

Number of	Beta FSH	Beta LH	Beta hCG	Beta TSH
-----	-----	-----	-----	-----
amino acids	92	118	115	145
carbohydrate groups	2	2	1	2 branched 4 straight
% carbohydrate	26	14 - 18	6 - 8	25 - 36
average Mol.Wt	14,500	16,500	15,200	22,200

The function of the carbohydrate moieties is still uncertain. They determine the biological half-lives of the hormones and therefore their biological activities in vivo, but they have little effect on in vitro assays. They contain sialic acid as important constituents. Differences in the sialic acid content account for differences in molecular weight of the hormone isolated

from different sources and differences in biological activity determined by in vivo assays (Brown, 1986).

The FSH and LH circulating in the blood are different from the hormones extracted from the pituitary gland. Moreover, the pituitary produces gonadotropins of different molecular weight according to the physiological state of the animal; the FSH and LH extracted from the pituitaries of oophorectomized rhesus monkeys had larger average molecular weights and longer biological half-lives than those obtained from intact females (Peckham and Knobil, 1976), attributed to changes in sialic acid content.

Purified FSH has also been prepared from the urine of postmenopausal women, the specific activity being 1255 iu/mg (Donini, 1976). Its molecular weight is only 28,000 daltons, it has 208 aminoacids, and has a biological activity of 780 iu/gm; this is different from pituitary FSH with a molecular weight of 41,000 daltons, and it has 254 aminoacids and its biological activity is 14,000 iu/gm. The aminoacid content shows differences from FSH derived from other sources as well. However, the aminoacid sequence and attachment of the carbohydrate moieties have not yet been reported (Brown, 1986); this information is required for the understanding of the biological activity of hMG used in therapy. The urinary FSH has a higher hexose and a lower sialic



acid content than the pituitary hormone. In spite of its dissimilarity with pituitary FSH, there is no clinically significant difference in ovarian stimulation when these preparations are given in biologically equivalent dosages (Schwartz and Jewelewicz, 1981).

The metabolic clearance rates (MCR) and serum half-lives of the gonadotropins have been studied by various groups. Because of the distribution of the hormones in more than 3 mathematical compartments, it was difficult to determine the true half-life (Kohler, Ross and Odell, 1968; Coble, Kohler, Cargille et al, 1969). The serum half-life of FSH has been reported at about 3 hours (Parlow, 1965), of LH from 1/2 to 1 hour (Pepperell, deKretser and Burger, 1975), and of hCG from 5 - 8 hours (Rizkallah, Gursipide and Van de Wiele, 1969). No figures could be found on the metabolic clearance rate and plasma half-lives of hMG preparations (Brown, 1986).

In contrast to treatment with clomiphene citrate (which requires an intact hypothalamus-pituitary), treatment with gonadotropins is independent of these structures and provides the total stimulus to the ovaries. Nevertheless, the majority of anovulatory patients have normal FSH levels, and therapeutic supplementation is to complement endogenous production so that



the total dose reaches the patient's threshold requirement for a follicular response. However, the dose requirement for FSH is critical, the difference between no-response and over-response being covered by a dose increment of less than 50%. Moreover, the dose required by different individuals may differ by 10-fold. This has resulted previously in frequent hyperstimulation and multiple pregnancies with inevitable fetal losses. Now, with step-wise increment of FSH from initial low doses and monitoring of ovarian response by blood or urine estrogens and ultrasound, such problems are less common.

Gonadotropin therapy requires 2 gonadotropin preparations, one to supply FSH for follicular maturation, and the other to supply LH for inducing ovulation of the matured follicle. Preparations supplying FSH include:

1. Human menopausal gonadotropin (hMG) - this is the main commercial source obtained from postmenopausal women's urine, and provides FSH:LH in the ratio of 1:1, each vial giving 75 i.u. FSH. The commercial preparations include Pergonal (Serono) and Humegon (Organon).
2. Purified human menopausal gonadotropin ("pure" FSH) - this has been recently introduced as Metrodin (Serono), and contains FSH:LH in the ratio of 100:1.
3. Human pituitary gonadotropin (hPG) - this is obtained from

cadaveric human pituitaries, and at extraction growth hormone is also obtained. The FSH:LH ratio varies from 1:1 to 1:3. Hence it is important to determine the exact ratio. Also the FSH concentration per vial may vary depending on the yield.

4. Pregnant mare's serum gonadotropin - contains largely FSH activity but cannot be used repeatedly in the human because of antibody formation. However, it is preferred over hMG for hyperstimulation of laboratory animals.

The LH preparation is hCG and is readily obtained from urine of pregnant women. hCG has a longer half-life than LH (see above) and is thus more economical for clinical use.

It was initially believed that pure FSH given in the follicular phase does not promote sufficient steroidogenesis, and that the LH component is necessary for it. The use of pure FSH was initially confined to patients with polycystic ovarian syndrome who have had normal or elevated levels of LH. However, only very recently has pure FSH been found to be able to stimulate the growth of good follicles (Bernadus, Jones, Acosta et al, 1984).

Hypogonadotrophic hypogonadal patients are the most suitable for gonadotropin therapy. Lunenfeld and Insler (1974) classified



such patients into 2 groups: group I - women with low basal gonadotropin levels and lack of endogenous activity (most of these patients have hypothalamic hypogonadotrophic hypogonadism); group II - women with low-normal or normal gonadotropin levels and evidence of estrogenic activity (most of these patients have hypothalamic-pituitary dysfunction and the polycystic ovary syndrome).

There are many regimes available. Thompson and Hansen (1970) summarized the various regimens:

1. Daily hMG, with hCG overlapping hMG for the last 1-3 days, and sometimes continuing for 1 or 2 days after stopping hMG.
2. Daily hMG, with hCG for 1 to 3 days starting 1 or 2 after hMG is stopped.
3. hMG on days 1,4 and 8 followed by hCG on day 11.
4. hMG alone.
5. hMG in a single dose followed by hCG on day 11.
6. hMG in combination with clomiphene.

Most centres now use an individualized regimen, and have abandoned all other methods. The most widely used method is as follows. Patients with no evidence of estrogenic activity are started on 2 ampoules of hMG daily while those with estrogenic activity are given 1 ampoule daily for the first 6 to 8 days.



Urinary or blood estrogens are monitored daily and ultrasound scans done to determine the number and size of follicles. If there is no ovarian response the dose is increased by 1 or 2 ampoules at variable intervals, from every day to once every 4 days. Once there is a response the dose is maintained until follicular maturation is attained. Ideally, follicular maturation is reached within 10 to 15 days of therapy. hCG is then given at doses between 3,000 to 10,000 iu after a variable "coasting" period (Brown, 1978). The luteal phase may have hCG or progestin support.

It is now evident that the reproductive hormones are secreted in a pulsatile manner (Lancet editorial, 1984). Hence it is logical to emulate the physiological patterns by pulsatile administration of gonadotropins. This method has been reported only recently (Ho Yuen, Pride and Sime, 1984) and experience with this approach has been limited compared to the pulsatile administration of GnRH.

Yet another new approach in gonadotropin administration is the use of large doses of a GnRH analogue (eg. agonist Buserelin, Hoechst) before exogenous gonadotropin administration. This has been reported for patients with poor luteal phase, for which the GnRH analogue was started during the luteal phase prior to the treatment cycle (Fleming, Adam, Barlow et al, 1982), and for

patients with increased LH and androgen levels, for which the GnRH analogue was administered 2-3 weeks prior to the treatment cycle (Fleming, Maxton, Hamilton et al, 1985).

Clomiphene may be combined with hMG. Clomiphene is given daily for 5 days either from day 2 or day 5. They are then continued on hMG for a variable period. With this regime there is a much higher chance of spontaneous LH surge (from 30 to 70% of cases). There may be advantages of anti-estrogenic effects of clomiphene especially against the high estrogenic levels caused by hMG.

Conceptions per cycle vary from 8 to 30% (Schwartz and Jewelewicz, 1981). Patients conceived vary from 16 to 60%. Outcome of pregnancies include an overall abortion rate of 28% and an overall multiple pregnancy rate of 28%, mostly the result of multiple ovulations.

The main complication of gonadotropin therapy is hyperstimulation. Rabau, David, Serr et al (1967) classified ovarian response after gonadotropin therapy into 3 main clinical categories and 6 grades:

Mild hyperstimulation:

Grade 1: Consists only of laboratory findings of hyperstimulation

- estrogen levels above 150 ug/24 hrs and pregnanediol excretion above 10 mg/24 hrs.

Grade 2: The above laboratory findings plus enlargement of ovaries; sometimes small cysts are palpable.

Moderate hyperstimulation:

Grade 3: In addition to above, abdominal distension is present.

Grade 4: In addition to above, vomiting and/or diarrhoea is/are present.

Severe hyperstimulation:

Grade 5: In addition to above, ovarian cysts are large and ascites and/or hydrothorax are present.

Grade 6: Marked hemoconcentration with increased blood viscosity may result in coagulation abnormalities.

Increased capillary permeability is probably the pathogenetic mechanism of severe hyperstimulation; the ascites is a transudate. The risk of hyperstimulation is related to the  $E_2$  level; however, if hCG administration is withheld, the hyperstimulation syndrome is prevented (Butler, 1969). It is interesting to note that in IVF patients, in spite of  $E_2$  reaching very high levels and hCG administration, hyperstimulation is uncommon; this may be probably due to the aspiration of follicles. Creation of



pneumoperitoneum may be a factor, in which case ultrasonic aspiration may be non-protective insofar as hyperstimulation goes. In our experience, in cases where the  $E_2$  levels were excessive (above 10,000 pg/ml) and follicles numerous (above 15 of average diameter more than 15 mm), there has been post-recovery ascites and severe enlargement of ovaries (unpublished data); there was no increase pre-disposition of ascites and ovarian enlargement in patients who had ultrasonic aspiration. Therapy with high FSH:LH ratio preparations causes less ovarian hyperstimulation (Crooke, Butt, Palmer et al, 1963); hence it is hopeful that pure FSH may minimise hyperstimulation. However, in our IVF and GIFT program such hyperstimulated cases were seen only with FSH:hMG stimulation; this was because it is with this regime that we had such high  $E_2$  values and large number of follicles. As these cases were in a later phase/series they were not included in the analysis of data. Mild hyperstimulation was seen in 8.4 to 23% of treatment cycles, moderate hyperstimulation in 6 to 7%, and severe hyperstimulation in 0.8 to 2% of cycles (Schenker and Weinstein, 1978).

### 3. Gonadotropin-releasing hormone (GnRH)

GnRH is a linear decapeptide [(Pyro) Glu-His-Trp-Ser-Tyr-Gly-Leu-

Arg-Pro-Gly-NH<sub>2</sub>] which binds to the specific receptors in the pituitary gonadotrophs. The binding sites for this hormone are located in the extracellular surface of the plasma membrane. The peptide evidently binds to the receptors with the glycine residue, which probably serves as a "spacer" to hold the more important amino acids in the proper spatial relationship for interaction with receptor binding sites (Stewart, 1981). Following the hormone receptor binding, adenylyl cyclase is activated, which in turn increases the amount of cyclic AMP (2nd messenger). The action of cyclic AMP on the synthesis of gonadotropins by pituitary cells is believed to involve various enzymes, such as protein kinase which phosphorylates various proteins. Furthermore, the ultimate and critical factor for the release of gonadotropins appear to be an increase in intracellular calcium (Blankstein and Lunenfeld, 1986).

GnRH has a very short half-life, due to inactivation at the Gly-leu bond by brain enzymes (Marks and Stern, 1974). It is a single neurotransmitter responsible for the secretion of both FSH and LH (Schally, Arimura and Kastin, 1971). Activation of GnRH-containing neurons depends upon neurotransmitters and sex steroids; there are interactions between dopamine, norepinephrine and GnRH in the nerve terminals of the hypothalamic median eminence (Zuspan and Zuspan, 1973). Gonadal



steroids alter the responsiveness of the pituitary to the action of GnRH. In the presence of low endogenous estrogens (early follicular phase of the menstrual cycle) the administration of GnRH results in minimal rise of both FSH and LH, while in the presence of high estrogens (late follicular phase) LH increases significantly while FSH increases only slightly. These patterns in the late follicular phase are not surprising, since in adult women a higher dose of GnRH is required to effect FSH release than for LH release (Yen, Lasley, Wang et al, 1975).

It was shown only recently by Belchertz, Plant, Nakat et al (1978) that pulsatile, not continuous, administration of GnRH was able to maintain pituitary gonadotropic function in rhesus monkeys which had their medio-basal hypothalamus ablated, thus abolishing their endogenous GnRH secretion. Hypothalamic amenorrhoea is considered as a defect of hypothalamic GnRH secretion. In women with severe hypothalamic amenorrhoea, pulsatile administration of GnRH at a frequency of 90 minutes resulted in follicular maturation, ovulation and corpus luteum formation (Leyendecker, 1979; Leyendecker, Struve and Plotz, 1980). Amenorrhoea as a symptom merely reflects a level of hypothalamic impairment within the pathophysiological entity of hypothalamic ovarian failure. Depending on the reduction of hypothalamic GnRH secretion, clinical pictures such as corpus luteum insufficiency,



anovulatory cycles, oligomenorrhoea, and finally, amenorrhoea may develop. Hence, corpus luteum insufficiency and anovulatory cycles develop when an insufficient dose of pulsatile GnRH is used to treat hypothalamic amenorrhoea (Leyendecker and Wildt, 1983). Secondary hypothalamic amenorrhoea is probably the result of the reduction of a pre-existing GnRH secretion and, functionally, a relapse of the pituitary-ovarian axis into the premenarchal state. This is often associated with more or less overt psychogenic or emotional stress (Fries and Nillius, 1973). Endogenous GnRH cannot be measured in peripheral blood and therefore direct evaluation of hypothalamic function is presently not possible and the diagnosis of hypothalamic amenorrhoea is essentially based on the exclusion of other causes of amenorrhoea, eg. hyperprolactinemia, hyperandrogenaemia, primary ovarian failure, genital tract defects, general systemic and neurological diseases.

Leyendecker and Wildt (1983) proposed the following grading of hypothalamic amenorrhoea on the basis of clomiphene, progestagen and GnRH tests:

Grade 1: A positive clomiphene test with menses after 100 mg/day for 5 days from the 5th day after withdrawal bleed to progestagen, indicates that there is only little impairment

of hypothalamic function. This is further sub-classified into: 1a) with normal luteal phase,  
1b) with insufficient luteal phase,  
1c) with anovulatory cycle.

Grade 2: Intermediate severity of hypothalamic amenorrhoea in which patients have progestagen-induced withdrawal bleed but do not bleed after clomiphene.

Grade 3: Severe hypothalamic amenorrhoeic patients who do not respond to progestagen withdrawal. They have differing response to a bolus dose of GnRH (100 ug), and are further subdivided into:

- 3a) "adult response" in which the increase of plasma/serum LH is more than FSH;
- 3b) "prepubertal response" in which the LH increase is impaired and is quantitatively and qualitatively similar to FSH;
- 3c) no response to GnRH, the most severe form of hypothalamic amenorrhoea.

The dose of GnRH is dependent on the degree of hypothalamic amenorrhoea. Thus patients with grades 2, 3a and 3b dysfunction ovulate and conceive with 2.5 - 5 ug/pulse, while patients with

grade 3c dysfunction need 15-20 ug/pulse before ovulating and conceiving. Individual and uncontrollable factors influencing the resorption of the hormone from the adipose tissue, however, reduce the ovulation rate when the subcutaneous route is chosen. This disadvantage can be overcome by increasing the dose to 15 - 20 ug/pulse for grades 2, 3a and 3b dysfunction with the subcutaneous route as used by most centres now.

Once the critical threshold of the GnRH dose is surpassed there seems to be a dose-response relationship between the dose of GnRH administered per pulse and the ovarian response, as reflected by E<sub>2</sub> and P response (Leyendecker and Wildt, 1983). Also, the duration of the follicular phase after pulsatile administration of GnRH is a reflection of the ovarian functional status at the beginning of the GnRH substitution. The average length of the follicular phase was 17 days in grade 3c, 14 days in grade 3b, 10 days in grade 3a and 9 days in grade 2 of hypothalamic amenorrhoea.

Decreasing the frequency of GnRH administration results in increased FSH and decreased LH plasma concentrations, thus affecting the FSH/LH ratio (Wildt, Hausler, Marshall, 1981). On the other hand, changes in amplitude of GnRH pulses have but minor effect on gonadotropin secretion (Wildt, Hausler, Marshall,



1981). However, in patients with enhanced pituitary sensitivity, ovarian hyperstimulation may occur (Schweditsch, Keller, Floer-sheim et al, 1984).

The normal luteotrophic hormone in the human is pituitary LH (Van de Wiele, Bogumil, Dyrenfurth et al, 1970). In severe hypothalamic amenorrhoea luteal function immediately ceases following termination of pulsatile GnRH substitution a few days after ovulation (Leyendecker and Wildt, 1983). Continuation of pulsatile GnRH during the whole luteal phase resulted in normal luteal phase. The luteal phase may also be supported by 1-3 injections of 2500 iu of hCG once ovulation has occurred (Weinstein, Seibel and Taymor, 1984).

GnRH can also be administered at 10 ug/pulse to normally cycling women to induce multiple follicular development (Liu, Durfee, Muse et al, 1983).

OVERSTIMULATION FOR IN-VITRO FERTILIZATION

1. Principle

The unifying goal of pregnancy underlies diverse strategies to monitor and/or control the ovulatory cycles of women undergoing

IVF and ET. This goal requires either stringent surveillance of an unperturbed spontaneous follicular phase, or hormonal regulation of the cohort. Edwards, Steptoe and Purdy (1980) credited their change from stimulated cycles to spontaneous cycles as the major breakthrough responsible for their first (Steptoe and Edwards, 1978) and subsequent pregnancies. They cautioned about the derangements of follicular steroidogenesis caused by ovarian hyperstimulation (Edwards and Steptoe, 1975). However, Trounson, Leeton, Wood, et al (1981) and Johnston, Lopata, Speirs et al (1981) proved that clomiphene citrate given daily from days 5 through 9 of the menstrual cycle within an IVF program could result in viable pregnancies.

The rationale of inducing maturation of several follicles for an IVF cycle is to maximise the number of recoverable oocytes. Most centres have successful retrieval of about 60 - 80% of follicles aspirated (Johnston, Lopata, Speirs et al, 1981; Renou, Trounson, Wood et al, 1981; Quigley, Wolf, Makland et al, 1982). Between 60 and 80% of these oocytes are fertilized, depending on the series and indications (Edwards, Fischel, Cohen et al, 1984; Mahadevan, Trounson and Leeton, 1983). However, less than 15% of these concepti result in implantations (Yovich, Stanger, Yovich et al, 1984a; Ng, Edirisinghe, Wong et al, 1986), and the recognizable pregnancy rate is less than 20%. However, increasing the



number of embryos replaced increases the chance of pregnancy (Speirs, Lopata, Gronow et al, 1983; Jones, Acosta, Garcia et al, 1983; Edwards, 1985; Seppala, 1985). Trounson (1983) noted a threefold increase in pregnancy rate when 2 embryos, rather than a single embryo, are replaced. Although the transfer of 3 or 4 embryos may enhance the pregnancy rate, there is an increase in the risk of multiple pregnancies (Biggers, 1981; Kerin, Warnes, Quinn et al, 1983b). It has been calculated that 2.5 accessible 15 mm follicles are necessary to ensure, on the average, transfer of 1 embryo to each patient (Quigley, Wolf, Makland et al, 1982); in fact, the authors recommended that patients with only one developing accessible follicle not undergo oocyte recovery during that cycle. With that policy, they have reported that when clomiphene citrate was used for follicular stimulation, only 4 of 96 patients (4.2%) had the laparoscopy postponed (Quigley, Makland and Wolf, 1983).

In principle the achievement of high FSH levels during the early follicular phase to recruit a maximum number of follicles with a tolerable degree of asynchrony for final maturation is confined to a limited time span of "FSH window" of about 3 to 4 days before negative estradiol feedback induces below-threshold FSH levels, thereby condemning all subsequent follicles in that cycle to atresia (Kerin, Warnes, Quinn et al, 1984). It appears that



one can widen and/or amplify the "FSH window" too far from gross hyperstimulation resulting in the recruitment of many follicles with an intolerable degree of asynchrony. This may lead to defective endocrine environment for the oocytes contained within these follicles or an abnormal luteal environment and an increase frequency of failure in fertilization, cleavage and implantation (Kerin, Warnes, Quinn et al, 1984).

## 2. Clomiphene

The earliest attempts in an IVF program to stimulate follicular development with clomiphene citrate and to effect oocyte maturation with hCG did not result in normal pregnancies. Edwards and Steptoe (1975) found no evidence of implantation in patients undergoing IVF after receiving clomiphene citrate at 100 mg/day from day 2 or 3 for 5 days, and 5,000 iu of hCG intra-muscularly on day 12 or 13. In another group of 27 patients treated with 50 to 100 mg clomiphene citrate for 5 days from days 3 to 11, 2 patients had a transient post-ER rise in hCG (Lopata, Brown, Leeton et al, 1978). In 1981, the Monash group (Trounson, Leeton, Wood et al, 1981) proved that clomiphene citrate could be used with good results in IVF when they reported 4 pregnancies induced with clomiphene, given at 150 mg daily from days 5 through 9. In

3 of the 4 women who became pregnant, 4000 iu of hCG was given 36 hours before laparoscopy predetermined by "follicle growth rate and availability of the operating room" (Trounson, Leeton, Wood et al, 1981). In a later report on 9 pregnancies induced with clomiphene citrate, 7 of these pregnancies (including 2 sets of twins) had progressed to between 16 and 31 weeks, while 2 aborted at 8 weeks (Wood, Trounson, Leeton et al, 1981). Other early reports of pregnancies induced with clomiphene supported the use of clomiphene in IVF (Johnston, Lopata, Speirs et al, 1981).

The maturational status of the retrieved oocyte in clomiphene-hCG stimulated cycles has been compared to that obtained during the monitored spontaneous cycle. The scheduling of laparoscopic oocyte recovery approximately 36 hours after hCG injection reduces the occurrence of pre-operative ovulation (Edwards and Steptoe, 1975) but may do so at the expense of invivo maturation. However, the recovery of mature, pre-ovulatory oocytes with cumulus masses indistinguishable from mature oocytes recovered in spontaneous cycles (Wood, Trounson, Leeton et al, 1981) argues for maintaining this hCG-laparoscopy interval. At laparoscopy, pre-ovulatory follicles in clomiphene-stimulated cycles appear identical to those in spontaneous cycles, with features suggestive of imminent ovulation (Wood, Trounson, Leeton et al, 1981). Testart, Frydman, De Mouzon et al (1983) that the average number



of retrieved oocytes with fully expanded, homologous cumulus masses did not differ significantly between spontaneous and clomiphene-stimulated cycles. However, there was an increase in the number of fertilizable, though less mature, oocytes exhibiting limited expansion of a heterogeneous, granular cumulus in the stimulated cycles (Testart, Frydman, De Mouzon et al, 1983).

Trounson and Leeton (1982) noted several disturbing anomalies in a small number of patients undergoing IVF who had clomiphene citrate. Some patients developed large cystic follicles which averaged approximately 3 cm in diameter and lacked a normal oocyte and normal granulosa cells; such follicles were already atretic before aspiration. Other patients experienced premature ovulation or lengthened follicular phases. However, those patients who had laparoscopic aspiration of follicles did not exhibit short luteal phases even though they were not supplemented with P. Normal luteal function in clomiphene-treated women undergoing follicular aspiration has been documented by Trotnow, Becker, Kniewald et al (1982) by monitoring BBT charts and plasma P levels.

Data of steroid concentrations from follicles in clomiphene-stimulated cycles has been confusing. Carson, Trounson and Findlay (1982) found that follicles from which oocytes were



retrieved contained significantly higher concentrations of both  $E_2$  and P compared with those from which no oocytes were found. Normal embryo growth following IVF was associated with significantly elevated levels of androstenedione, P and  $E_2$ , while pregnancy was correlated with significantly higher levels of  $E_2$ . They found that only follicles with high P levels produced healthy, fertilizable oocytes. Another group of workers also correlated in spontaneous cycles fertilizability of collected oocytes with high follicular concentrations of  $E_2$  and P (Wramsby, Kullander, Liedholm et al, 1981). Testart, Frydman, Castanier et al (1983) also reported on follicular fluid steroids in spontaneous and clomiphene-stimulated cycles. Treatment with 100 to 150 mg clomiphene on days 5 through 9 reduced the proportion of follicles with normal steroid concentrations. Most of the abnormal follicles had high androgens or low P concentrations. They also found that in spontaneous cycles, the success rate in IVF was not significantly influenced by follicular steroid levels provided minimum values were achieved; this was in contrast to the findings of Wramsby, Kullander, Liedholm et al, (1981). Based on these conflicting findings, follicular fluid steroid concentrations may not be predictive of the outcome of either spontaneous or clomiphene-stimulated cycles in IVF.

Follicular fluid concentrations of gonadotropins also do not of-

fer any predictive value in IVF outcome. Testart, Frydman, Castanier et al (1983) noted FSH values  $\geq 0.3$  mIU/ml in all the pre-ovulatory follicles aspirated during spontaneous cycles, compared with only 75.9% of clomiphene-stimulated (and hMG) cycles. The FSH concentration did not influence the dissociation of cumulus (Testart, Frydman, De Mouzon et al, 1983).

There is also the question of dosage. Trounson and Leeton (1981) reported that 50 mg clomiphene citrate administered daily on days 5 through 9 did not produce more than 1 large follicle. However, in a study comparing clomiphene 50 mg and 150 mg/day from day 5 through 9, Quigley, Makland and Wolf (1983) reported that there was no statistical differences in the number or size of follicles as determined by ultrasound on the day of hCG administration between the 2 groups. The mean number of follicles was  $2.3 \pm 0.1$  in the 50 mg group and  $2.4 \pm 0.2$  in the 150 mg group. There was no difference in oocyte recovery and their outcome between the 2 groups (Schmidt, 1984), and in greater than 90% of all treatment cycles, patients underwent laparoscopy. The oocyte recovery rate was around 60%, with the fertilization rate around 70% and the cleavage rate around 95%. The only statistical difference was the higher clinical pregnancy rate in the 50 mg clomiphene group (19.4% per ER) as opposed to none in the 150 mg clomiphene group. This study illustrates the possible negative outcome in increas-



ing the ovulatory stimulation.

Fixed schedules with clomiphene have been used to collect oocytes for research purposes from volunteers requesting laparoscopic sterilization (Braude, Bright, Douglas et al, 1984; Templeton, Van Look, Lumsden et al, 1984; Messinis, Templeton, Angell et al, 1986). The various authors used varying doses and duration of clomiphene to induce multiple follicular development, and Messinis, Templeton, Angell et al (1986) reported that there was no difference in the number of oocytes collected with clomiphene administered at 50 mg and 150 mg daily for 5 days and 50 mg daily for 10 days. The patients were admitted on day 16 and 1400 iu of hCG was administered at 0200 hours on day 17. The laparoscopy was arranged for midday on day 18 (around 34 hours after the hCG). Oocytes were recovered from 83% of the aspirated follicles (1.6 oocytes/patient); 65% of the oocytes cleaved after IVF and, on the average, 1.0 cleaving egg was obtained per patient. However, as there were no embryo replacements and therefore no pregnancies, it was not possible to comment whether the oocytes or embryos obtained were developmentally normal.

When the patient had only one ovary, clomiphene stimulation resulted in a higher initial  $E_2$  level and a greater number of follicles  $\geq 15$  mm, but there was no difference in the number of



oocytes collected from patients with one or two ovaries (Diamond, Wentz, Herbert et al, 1984). The authors also reported on similar findings with hMG stimulation, but the number of oocytes collected were more from patients with 2 ovaries. In another study comparing response from patients with one or two ovaries, stimulation with clomiphene-hMG did not result in any difference in mean number of follicles  $\geq 15$  mm, oocytes collected, embryos transferred and resulting pregnancies (Alper, Seibel, Oskowitz et al, 1985).

Finally, there is the question of brand, or make, of the clomiphene. Diamond, Herbert, Maxson et al (1986) compared clomiphene from Merrell Dow (Clomid) and from Serono (Serophene) using the same dosage 150 mg/day from day 3 through 7. The only difference detected in response was a greater elevation in  $E_2$  levels with Clomid; no differences were detected in laparoscopies, eggs recovered, fertilization and embryo development.

### 3. hMG & other gonadotropins

The initiation of follicular growth is thought to be dependent on factors in addition to gonadotropins (Schmidt, 1984). However, FSH is essential to: (1) increase the number of granulosa cells; (2) increase the number of FSH and later LH receptors on

granulosa cells; and (3) increase the aromatase activity of these granulosa cells. The main purpose of FSH is to stimulate follicular growth to the pre-ovulatory stage and to facilitate conversion from an androgenic to an estrogenic follicular environment (Beauchamp, 1984).

The mechanism determining follicular selection and atresia may be influenced by altering the gonadotropin patterns of the menstrual cycle. By preventing the decline in FSH (or actually increasing its level) while follicular selection and dominance is occurring, several extra follicles may be rescued from atresia and go on to develop to the pre-ovulatory stage. These increased gonadotropin levels may be accomplished directly with hMG administration or indirectly by clomiphene citrate or GnRH administration.

Ovarian response to hMG administration is monitored by clinical, ultrasonographic and laboratory methods. The size of pre-ovulatory follicles, as determined by ultrasound, is smaller with hMG stimulation when compared to clomiphene-stimulated or natural cycles (Renaud, Macler, Ehret, et al, 1983; Mantzavinos, Garcia and Jones, 1983; Buttery, Trounson, McMaster et al, 1983). A mature, fertilizable oocyte can usually be obtained from 15-17 mm diameter follicles during hMG stimulation (Garcia, Jones, Acosta et al, 1983). In clomiphene stimulated or natural cycles, mature



oocytes are usually obtained only from follicles  $\geq 20$  mm in diameter. Possible explanations for this discrepancy are either (a) inhibition of endogenous LH by hMG; (b) inhibition of follicular growth by a fixed FSH/LH ratio during hMG therapy; (c) accelerated oocyte maturation with hMG; or (d) decreased number of granulosa cells or follicular fluid volume with hMG (Beauchamp, 1984).

The circulating levels of  $E_2$  are derived primarily from developing follicles (Fritz and Speroff, 1982). Measurements of serum or urinary  $E_2$  concentrations have been utilized to monitor follicular development in patients undergoing ovulation induction. Studies have shown that, during hMG stimulation, plasma  $E_2$  levels correlated well with total follicular volume (Haning, Austin, Kuzma et al, 1982). It was also reported that in hMG-stimulated cycles, there were significantly elevated  $E_2$  levels in follicular fluid from women who subsequently became pregnant (Botero-Ruiz, Laufer, deCherney et al, 1984). Three patterns of estrogen response to hMG stimulation have been identified: low, normal, and high estradiol responses corresponding to  $E_2$  levels of  $< 300$ ,  $300-600$ ,  $> 600$  pg/ml respectively (Jones, Acosta, Andrews et al, 1983).

Studies on anovulatory women undergoing ovulation induction with



hMG show that the duration of the active phase of follicular maturation and the rate of exponential  $E_2$  rise may be as important as absolute  $E_2$  levels in determining normalcy of the luteal phase, conception and multiple pregnancy rates (Berquist, Nillius and Wide, 1983a & 1983b). Even though the ovulatory dose of hCG was given at a standard  $E_2$  level (2000 pmol/l), a short active phase (7 days from baseline to threshold levels) was associated with a defective luteal phase of anovulation. Furthermore, conceptual cycles were associated with lower  $E_2$  levels during the follicular phase and higher P levels during the luteal phase. It appears that rapid follicular development by hMG (as evidenced by rapid  $E_2$  rise), results in lower pregnancy rates and abnormal luteal phases probably due to ovulation of submature oocytes or abnormal corpus luteum formation (Yuen, Sy and Cannon, 1981).

Duration of the hMG administration to anovulatory women (number of days necessary to increase  $E_2$  from baseline to threshold levels) may also influence follicular recruitment. Singleton pregnancies were associated with treatments of  $\leq 7$  days duration while multiple pregnancies had longer (8-10 days) active phases. It appears that the longer ovarian exposure to hMG predisposes it to multiple follicular development (Berquist, Nillius and Wide, 1983a).

The ovarian response to hMG depends on at least 2 factors: the amount of gonadotropins administered and the state of the follicles at the beginning of therapy (Insler and Potashnik, 1983). Each patient appears to have her own sensitivity to hMG, accounting for the variation observed in  $E_2$  response and in follicular development.

hMG has been used alone for follicular recruitment in IVF programs; this has been pioneered mainly by the Eastern Virginia Medical School. Initially they used a protocol similar to that used for induction of ovulation (Garcia , Jones, Acosta et al, 1983a). Two ampoules of hMG (150 iu FSH and 150 iu LH) was administered starting on the first, third or fifth day of the cycle depending on cycle length, < 25 days, 25-35 days, or > 35 days, respectively. After 3 days of treatment, the dose was lowered to 1 ampoule of hMG daily. With the appearance of the "estrogen clinical shift" (defined as  $\geq 30\%$  superficial cells on vaginal cytology, cervical mucus volume  $\geq 0.2$  ml, spinnbarkeit  $\geq 10$  cm, and 4+ ferning), hMG was discontinued and 10,000 iu hCG was administered 2-3 days later, depending on when the largest follicle reached 18 mm in diameter as determined by ultrasound. There was no endogenous LH surge in this study. Oocytes were recovered from all patients except one (31 cycles in 25 patients) with 60 oocytes recovered in all (1.94 oocytes/cycle). Eighty % of those



oocytes were pre-ovulatory, 15% were immature and 5% atretic. Only 16 of the 48 (33%) pre-ovulatory oocytes fertilized and cleaved, and 2 pregnancies resulted from embryos transferred to 12 patients (Wortham, Veeck, Witmyer et al, 1983).

During the second phase (II) of their program, the hMG protocol was individualized according to the estrogen level at the time of the clinical estrogen shift (Garcia, Jones, Acosta et al, 1983b). Two ampoules of hMG were administered starting on the third or fifth day in patients who had  $28 \pm 3$  and  $35 \pm 3$  days cycles, respectively. Further management was based on serum  $E_2$  levels and the occurrence of the "clinical shift". For low responders ( $E_2 < 300$  pg/ml on the day of the clinical shift) hMG was continued for 3 additional days after the shift. In the normal  $E_2$  responders ( $E_2 300 - 600$  pg/ml) hMG was discontinued if the shift had occurred. In the high estrogen responders, hMG was discontinued once the  $E_2$  exceeded 600 pg/ml, even if the clinical estrogen shift had not occurred. The ovulatory dose of hCG (10,000 iu) was administered 50 hours after the last hMG injection (Jones, Jones, Andrews et al, 1982). A normal  $E_2$  response was observed in 17 patients, 3 patients had a low and 4 had a high  $E_2$  response.

These 24 patients underwent laparoscopy. No oocytes were obtained in 1 patient and only immature oocytes were obtained in another.



Ninety-eight follicles were aspirated (4.1 follicles per patient) and 57 oocytes were recovered (2.4 oocytes per patient). Forty oocytes were mature (70%). One patient had one follicle ruptured prior to laparoscopy but mature oocytes were recovered from other follicles. Of the mature oocytes 30 out of 33 fertilized and cleaved; 7 of 10 immature oocytes matured in vitro, fertilized and cleaved. Five pregnancies resulted after transfer of 37 embryos to 19 patients.

Besides an overall improvement in outcome, there were 2 differences between phases I and II. First, oocyte post-maturity (aging) was observed in 7 patients during phase I. Fragmentation of the oocytes and darkening of granulosa cells (lipid inclusions from excessive luteinization) were observed, most frequently in high responders. This observation was attributed to the result of prolonged hMG stimulation and failure of the  $E_2$  from the developing follicles to elicit an endogenous LH surge. Post-maturity was decreased by shortening the duration of hMG therapy and by shortening the interval from the last hMG dose to the time of hCG administration to 26 hours in the high  $E_2$  responders.

Secondly, serum P levels were 3 times higher at the time of hCG injection and laparoscopy in the high  $E_2$  responders compared to normal or low responders. Higher serum P values were seen at the

time of laparoscopy in follicles with dark granulosa cells and fragmented oocytes. It appears that excessive, premature elevation of P values (seen mainly in high responders) is detrimental to the follicle and oocyte.

In further analyses of their results with the second stimulation regime, Jones, Acosta, Andrews et al (1983) concluded that the best pregnancy rates were from the high responders. In 175 consecutive patients who underwent laparoscopy, 26 were high responders, 109 normal responders, and 40 low responders. The number of follicles per cycle were 5.5, 4.26 and 4.1 for high, normal and low responders, respectively. An average of 1.69, 1.47 and 1.05 preovulatory oocytes were obtained per cycle producing a pregnancy rate of 23%, 19% and 15% for the high, normal and low responders.

Relatively high doses of hMG had also been used successfully for IVF (Laufer, deCherney, Haseltine et al, 1983). The authors reported a mean of  $19 \pm 4$  ampoules of hMG per cycle were used, starting from day 3 at 3 ampoules/day. However, in 55 laparoscopic oocyte recoveries, a mean of 4.3 follicles were aspirated, and 3.2 oocytes/patient was recovered. An average of 3.5 embryos were transferred, and 17% conceived. In spite of such high hMG doses, 8% of the cases did not respond adequately.



There was a positive correlation between body weight, length of hMG stimulation, and oocyte fertilization rate (Halme, Hammond, Talbert et al, 1986). One hundred and four normally cycling women were treated with a fixed low-dose hMG regimen (2 amp/day) from day 3 till serum  $E_2$  was above 400 pg/ml or at least 2 follicles  $\geq 14$  mm in largest diameter were measured by ultrasonography. The response rate was found to be related to age, weight, and the ratios weight/height<sup>2</sup> and weight/height. The required dose of hMG was related to total body weight, but not to age or either of the weight/height ratios. In patients who require fewer than 5 days of hMG (rapid responders), a significantly higher number of oocytes were harvested, compared with slow responders. However, fertilization rate was less for the rapid responders, and this resulted in a reduced embryo transfer rate. The pregnancy rates were 11% and 17% for the rapid and slow responders respectively (not significant). The authors suggested that patients undergoing IVF who weigh less than 55 kg may benefit from treatment with a lower dose of hMG to prolong the stimulation cycle.

The use of purified FSH (Metrodin, Serono) in combination with hMG has been reported by Bernadus, Garcia, Jones et al (1984), Muasher, Garcia, Rosenwaks (1985) and Jones, Acosta, Garcia et al (1985a). Rosenwaks, Muasher and Acosta (1986) reported that



purified FSH was used in the stimulation of "poor" responders; of 12 such patients treated, 8 were adequately stimulated, and 5 of them became pregnant. However, after trying various combinations of purified FSH with hMG and pure FSH alone in IVF, Jones (1986) reported that protocols with a shifting ratio of FSH to LH gave slightly better results than FSH alone. Moreover, protocols with ratio of FSH to LH greater than one had a statistically better pregnancy rate. FSH administered without LH induces estrogen synthesis similar to that induced by equal amounts of FSH and LH, and increased numbers of follicles and oocytes with apparently normal pregnancy potential (Channing, Liu, Jones et al, 1983). The administration of FSH only is associated with somewhat higher estrogen values, prior to discontinuance of FSH stimulation, and slightly larger follicles than those seen with FSH/LH stimulation (Jones, Acosta, Garcia et al, 1985b). These factors must be considered in monitoring "pure" FSH cycles.

Another method of administering gonadotropins for stimulation of multiple follicles for IVF is by pulsatile injections. Afnan, Hillier, Margara et al (1984) reported on a successful IVF attempt resulting in a clinical pregnancy; after clomiphene administration from days 3 to 7, 2 ampoules of hMG daily was administered i.v. every 90 minutes on days 8 to 10 through a pump. There were 2 follicles on her left ovary and 4 on her right.

Seven oocytes were collected and 6 fertilized.

It is generally accepted that during a normal menstrual cycle ovulation blocks further maturation of additional follicles, either locally, centrally, or both. The mid-cycle progesterone rise is implicated in both mechanisms (Fritz and Speroff, 1982). With ovulation induction, especially with gonadotropins, multiple ovulation leading to multiple pregnancies is a common occurrence. The probability of asynchronous ovulation during ovulation induction was suggested by the recovery of multiple ova at different stages of maturation in IVF programs. In fact, there was a recent case report of multiple ovulations in a gonadotropin-induced cycle occurring within an interval of 110 hours from the time of commencement of the mid-cycle surge triggering the first ovulation to the time the last Graafian follicle was about to rupture (Navot, Margalioth, Laufer et al, 1984). One egg was recovered from a ruptured follicle, and it fertilized and cleaved to 4-cell.

#### 4. Clomiphene with hMG

There have been a few studies comparing clomiphene citrate, clomiphene citrate augmented with hMG, clomiphene citrate followed by hMG, and hMG alone. Vargyas, Morente, Shangold et al



(1984) compared 4 protocols: Group I consisted of clomiphene 150 mg per day from days 3-7; group II received clomiphene 150 mg/day from days 5-9 followed by 2 ampoules of hMG from 3-5 days until adequate follicular development; group III patients received clomiphene 150 mg/day from days 3-7 as well as 2 ampoules hMG on days 3, 5, 7, 8, 9, 10 and 11 of the cycle; and group IV received 2 ampoules of hMG starting on day 3 and extending 6 to 8 days until adequate follicular maturation had occurred. hCG 4000 iu was administered on the day ultrasound revealed at least one follicle  $\geq 18$  mm diameter.

The mean number of follicles per patient was 3.3, 6.0, 4.5 and 4.3 for groups I, II, III and IV, respectively. These numbers were significantly higher for group I. The mean number of embryos transferred per patient was 2.2, 3.0, 2.4, 1.5 for groups I, II, III and IV respectively. Pre-ovulatory  $E_2$  levels were lower in groups I and IV. Fertilization rates were highest in groups II and III, and slightly lower in groups I and IV. Only 3% of group II patients had a spontaneous LH surge prior to hCG as opposed to 33% of group IV patients. The best overall results were observed in group II (clomiphene followed by hMG) followed by group IV (hMG alone), though the numbers were small.

Quigley, Schmidt, Beauchamp et al (1984) reported a randomized



study which compared clomiphene 50 mg/day from days 5-9 of the cycle (group I), with clomiphene-hMG combination where the clomiphene was given as before and 2 ampoules of hMG on days 6, 8 and 10 (group II). The mean number of follicles per patient were significantly higher in group II (5.1 vs 3.7). There was a higher number of follicles 10-14 mm in size seen in group II; however, no difference was noted in number of follicles from 15 mm diameter. Oocytes (mature and immature), fertilization and cleavage rates were similar in both groups. More embryos were transferred in group II (2.5 vs 2.0), but this was not significant. There were 2 clinical pregnancies in 12 transfers in group I, and 2 pregnancies in 13 transfers in group II. The authors believed that clomiphene plus hMG (in combination or in sequence) appears better than clomiphene citrate alone.

In a very large comparative series, Trounson and Wood (1984) reported on the stimulation with clomiphene in 418 patients, hMG alone in 21 patients, and clomiphene-hMG combination in 364 patients, together with 71 unstimulated patients. There were 160 embryo replacements in the clomiphene group, 5 in the hMG group, and 224 in the clomiphene-hMG group; the pregnancy rates were 21.25%, 60.0%, and 19.2% respectively. However, there was no data on the average number of follicles, eggs, embryos and E<sub>2</sub> response in the 3 groups.

In another recent report on comparison of various stimulation regimes (Diamond, Hill, Webster, et al, 1986), hMG was given at 2 or 3 ampoules/day from day 3 for 2 days, then 2 ampoules/day until maturity (group I - 181 patients); clomiphene was given at 150 mg/day from days 3-7 (group II - 42 patients); hMG for the clomiphene-hMG combination was 2 ampoules/day from day 6 or 7 (group III - 81 patients). The ovulating dose of hCG was 4000 iu. Patients undergoing laparoscopic recovery were not significantly different in the 3 groups (69%, 71%, and 74% respectively), nor were the rates of oocyte recovery (94%, 100%, and 100%). However, the percentage of women achieving fertilization (77%, 83% and 93%) and embryo transfer (73%, 83%, and 90%) were significantly greater in group III (clomiphene-hMG combination). However, there was no increase in pregnancy rates in the combined regime.

The amount of clomiphene given before the hMG stimulation did not affect the follicle numbers or fertilization rates (Bayly, McBain, Clarke et al, 1985). 283 patients had clomiphene 100 mg/day, with hMG from the fifth day of clomiphene until maturity; 85 patients had similar stimulation but with reduced clomiphene at 50 mg/day. There was no difference in number of hMG ampoules (12 ampoules each), days of hMG (4.3 and 4.4 respectively), and cycle day of oocyte recovery (day 14.3 for both). Peak  $E_2$  values



were 1,900 pg/ml for both, with 4.4 and 4.5 eggs per recovery respectively, 2.1 and 2.2 embryos per recovery respectively, and pregnancy rates at 17.7% and 14.1% per recovery respectively. The only significant difference was the lower incidence of spontaneous LH surges in the group with higher clomiphene dose (20% versus 34% respectively).

When the hMG is given in a combined clomiphene-hMG protocol it has different effect on the outcome. In a unique cross-over trial involving the two main IVF centres in Melbourne, Rogers, Molloy, Healy et al (1986) reported higher pregnancy rates with the Monash protocol. The Monash protocol utilized the calculation of the predicted day of the spontaneous LH surge based on 6 previous cycles, and clomiphene was given at 100 mg/day starting 10 days before the predicted surge; hMG was given at 2 ampoules/day starting 9 days before the predicted LH surge, and continued till the  $E_2$  response was between 500 and 1000 pg/ml and showed a steady rise. The other centre, the Royal Women's Hospital (RWH), started clomiphene (100 mg/day) between days 3 and 5 depending on the cycle length; hMG at 3 ampoules/day was started when the  $E_2$  showed a positive rise (of above 1.5 times previous mean) and continued for 4 days. There was no difference in amount of hMG given. The average number of oocytes per patient were 3.4 for the Monash patients on RWH protocol (Monash control 3.8), and 7.0 for



the RWH patients on the Monash protocol (RWH control 5.2). Pregnancy rates were 8% for Monash patients on RWH protocol (Monash control 15%), and 30% for RWH patients on Monash protocol (RWH control 21%). It does seem that the Monash protocol yielded better results, though that was not statistically significant.

It has been reported that use of clomiphene with hMG for IVF resulted in high LH levels in peripheral blood (Jeffcoate, 1985). Such high LH levels may cause premature activation of the egg which is capable of fertilization and cleavage, but fails to implant; excess LH may cause down-regulation of LH receptors with abnormalities in the follicular response to the LH surge and defects in luteal function. It may also cause premature luteinization of the granulosa cells with resulting P production. Such abnormal P production may cause asynchrony between embryo stage and endometrium; it may also increase PRL production with consequent disruption in ovulatory response.

Okamoto, Healy, Howlett et al (1986) reported the 5th and 95th percentile envelope of plasma  $E_2$  concentrations from 102 consecutive IVF conceptions after stimulation with clomiphene-hMG. The mean  $E_2$  levels on the day before the calculated mid-point (anticipated LH surge) at the 50th, 5th and 95th percentile were 1656 pg/ml, 740 pg/ml and 3638 pg/ml respectively. The plasma  $E_2$

range reported defined objectively the diagnosis of ovarian hyperstimulation and inadequate stimulation in an IVF program. A new approach in the monitoring of follicular stimulation is the use of salivary  $E_2$ ; this may obviate multiple venepunctures for patients (Belkien, Bordt, Moller et al, 1985).

#### 5. Use of GnRH or their analogues for IVF

In order to lower the LH levels associated with clomiphene-hMG cycles, and to increase the number of follicles from the same cohort, attempts have been made on suppressing endogenous gonadotropins with GnRH analogues, usually Busserelin. There were a few such reports presented in the 4th World Congress on IVF held in Melbourne, November 1985 (Bordt, Belkien, Hanker et al, 1986; Porter, Smith, Craft et al, 1986; Pring, Setchell, Quinn et al, 1986; Sharma, Riddle, Williams et al, 1986). There were large numbers of oocytes collected after stimulation with either hMG or purified FSH, and because they were of different maturity they were obviously from different cohorts. Until the full papers are published it is rather difficult to comment on their use.

#### 6. Use of other agents for control of stimulation in IVF



In an attempt to synchronise ovulation, and therefore simplify and improve on logistic planning for an IVF program, Frydman, Forman, Rainhorn et al (1986) reported on the use of a triphasic estrogen-progestin contraceptive pill or a progestin (norethisterone) on the cycle before the planned IVF cycle. Following this "pre-treatment" (day 0 was the day the pre-treatment ended) the patient was stimulated with clomiphene 100 mg/day from days 2 through 6, and hMG 2 ampoules/day on days 2, 4, 6, 8 and 10. hCG (5000 iu) was administered on day 11 and egg retrieval was done on day 13 (35 hours after the hCG). No hormonal or ultrasonic monitoring was done. In 35 cycles compared with 34 control cycles, there were 3.9 and 4.8 follicles at egg retrieval respectively, 2.7 and 3.2 oocytes recovered/retrieval respectively, 2.0 and 2.4 embryos/retrieval respectively, and 8 and 4 clinical pregnancies respectively. Because there was no improved pregnancy rate in the control group, the authors suggested that the usual monitoring methods are suboptimal, being unable to define the subgroup of patients to proceed to aspiration who were more likely to develop a clinical pregnancy than an unmonitored population. Also, they were concerned with identifying the treatment schedule associated with the highest incidence of high responders. However, they felt that their preliminary results justified adopting a fixed schedule ovulation regimen for their patients.



Another approach is the use of pulsatile GnRH to stimulate folliculogenesis. Multiple follicles can be induced with pulsatile GnRH therapy (Bogchelam, Lappohn and Janssens, 1982; Liu, Durfee, Muse et al, 1983). Hence it is possible to use this approach for IVF stimulation. This has been reported by Kerin, Broom, McEvoy et al (1984) who used subcutaneous pulsatile GnRH for stimulation but the response was unsatisfactory.

Patients with incipient ovarian failure, with elevated FSH in the early follicular phase and normal LH, characteristically do not respond to hMG treatment, or they may have a rapid response with a premature LH surge (Jones, Muasher, Rosenwaks, et al, 1986). To overcome these problems, the authors administered intravenous pulsatile GnRH 5-10 ug/90-120 min to 8 such patients. Two patients failed to respond with follicular maturation. Another 4 could have had unrecognised LH surge; 3 had no oocytes aspirated from apparently postmature follicles or had postmature oocytes, and 1 had treatment cancelled due to ovulation. In the remaining 2 patients, one oocyte was fertilized and transferred, and one pregnancy resulted.

## 7. Spontaneous LH surge

In a clomiphene-hMG study which compared 8 patients who had spontaneous LH surge with 7 patients without spontaneous LH surge (Nader, Berkowitz, Maklad, Wolf et al, 1986), there was a significantly higher  $E_2$  per follicle  $\geq 15$  mm on the morning of the hCG administration or LH surge in the LH surge group. However, non-surge patients had a greater number of follicles  $\geq 15$  mm. The authors believe that greater quantities of nonsteroidal hormones, eg inhibin, produced by a greater number of preovulatory follicles in nonsurge patients, may block the pituitary response to hypothalamic GnRH in the presence of high and rising  $E_2$ , thus preventing the spontaneous LH surge.

In a later report, the authors looked at the  $E_2$  pattern in the patients with spontaneous LH surge (Nader, Berkowitz, Ochs et al, 1986). They distinguished 2 patterns - a "leap" pattern, where the rate of  $E_2$  rise increased progressively until peak  $E_2$  was reached, or when the change in  $E_2$  between the peak and the previous day's value exceeded 300 pg/ml; a "plateau" pattern, where the rate of rise declined as the LH surge approached or where the change in  $E_2$  between the peak and the previous day's value was  $\leq$  100 pg/ml. Of the 16 cycles, 9 had "leap" patterns and 7 had "plateau" patterns. There was no difference between the groups in the peak  $E_2$  attained, and the number of follicles  $\geq 12$  mm on the day of peak  $E_2$ . However, when the number of follicles  $\geq 12$  mm



ations of follicles. An inadequate luteal phase results because there is a direct relationship between the number of small, unripe follicles at the time of follicular aspiration, and the existence of a "relative" deficiency of P which can be detected in the luteal phase (Lehman, Diedrich, van der Ven et al, 1984).

Excessive exogenous gonadotrophins may also result in hypertrophied aggregates of smooth endoplasmic reticulum in the cytoplasm of the egg (Sathananthan, Ng, Ratnam et al, 1986). Such morphological change is indicative of excessive steroidal output; while this association is clear in granulosa cells, its significance in the egg is still uncertain.

Finally, there is a possibility that there may be an increase in chromosomal abnormalities as a result of excessive stimulation. The proportion of human oocytes with chromosomal abnormalities is not yet known. However, recently Wramsby, Fredga and Liedholm (1987) reported that infertile women undergoing clomiphene stimulation have a high proportion of oocytes (nearly 50%) with an abnormal karyotype. It may be that the more abnormal forms of oocytes that would normally undergo atresia were "rescued" by ovulatory drugs. If so, then overstimulation with gonadotropins would result in more oocytes, and hence more abnormal oocytes.



CHAPTER 3:

MATERIALS AND METHODOLOGY

PATIENTS

When the IVF program was in progress at the Kandang Kerbau Hospital, there was no GIFT program available. Hence the indications for patients for inclusion into the IVF program were:

- 1. tubal blockage or disease in the female
- 2. male infertility: oligospermia or oligoasthenospermia
- 3. idiopathic subfertility
  - (i) subfertility of more than 5 years in female patients below 30 years of age;
  - (ii) subfertility of between 2 and 5 years in female patients above 30 years of age.
- 4. miscellaneous (immunologic infertility and endometriosis)

In phase I (July 1982 to May 1983), the patients were either unstimulated or were superovulated with clomiphene citrate (Clomid, Merrell). Twenty-two patients were involved in 33 patient cycles (Ng, Ratnam, Law et al, 1984). Their ages ranged from 25 to 39, with a mean of 31.9 years. Thirteen patients had

tubal obstruction, 4 had oligospermia, 4 had idiopathic subfertility, and 1 had endometriosis (table 3.1).

Phase II (September 1983 to September 1985) included 100 patients in 105 cycles, with 100 laparoscopies (table 3.1). Their ages ranged from 29 to 40, with a mean of 32.9 years. The majority of patients had tubal obstruction (48.0%), while 22.0% had a combination of indications (table 3.1).

#### STIMULATION REGIME: PHASE I

Superovulated patients were orally administered 50 mg of clomiphene citrate (Clomid, Merrell) three times a day for 5 days commencing on day 2 (Ng, Ratnam, Law et al, 1984). Plasma  $E_2$  levels were monitored from day 8 onwards. Ultrasound scans were initially performed on day 9, but later carried out daily from day 8 onwards (see chapter 9). When the dominant follicle had at least a diameter of 1.5 cm or more, the patient was admitted. Three-hourly urine samples were assayed for LH. In the absence of an endogenous LH surge, 5000 iu of hCG (Pregnyl, Organon) was given intramuscularly when the dominant follicle had an obvious cumulus oophorus or a diameter of 2.0 cm or more.

Daily venous samples were obtained from the antecubital vein between 8 and 9 am. Unconjugated plasma  $E_2$ -17B was measured by radioimmunoassay, using rabbit antiserum to 6,7-3H-17B estradiol with a total incubation time of about 2 hours. The 3-hourly urine samples were collected throughout the 24 hours, with fluid intake restricted to 1.5 litres per day. LH was measured by a haemagglutination inhibition kit method, Higonavis (Mochida Pharmaceuticals, Tokyo), twice daily. The details of this method are given in chapter 8.

#### STIMULATION REGIME: PHASE II

Fifty mg of clomiphene was orally administered twice a day from day 2 for 5 days. On day 6, hMG (Pergonal, Serono) was injected. In regime I, one ampoule (75 i.u. of FSH and 75 i.u. of LH) was given daily from day 6 to day 8, while in regime II, 2 ampoules (150 i.u. of FSH and 150 i.u. of LH) were injected each day for the same duration (Ng, Ratnam, Law et al, 1985a). From day 9, the hMG dosage was dependent on the plasma  $E_2$  levels and taken daily at 8.30 am (see chapter 6). If the  $E_2$  value was less than 150% of the previous day's level, 1 ampoule of hMG was added to the previous day's dose. If the  $E_2$  value was more than 200% of the previous day's level, 1 ampoule was deducted from the previous day's



dose. If the level was between 150 to 200%, the dose was maintained. All hMG injections were given at 4.00 pm. An ultrasound scan was carried out on day 9 to determine the number of the follicles. No repeat scan was done unless necessary as it interfered with the urinary LH monitoring. Moreover, recruitment of follicles after day 9 has been reported to be rare (Blankenstein, Saadon, Mashiah et al, 1984). The patient was admitted when the  $E_2$  level reached 400 pg/ml/follicle. Urine was then collected 2 to 4 hourly, and LH values determined with Hi-Gonavis (Mochida, Tokyo) twice daily by the modified method of Edwards, Anderson, Pickering et al (1982). In the absence of the LH surge, 10,000 i.u. of hCG was given when the plasma  $E_2$  level reached either 500 or 700 pg/ml/ml; a "coasting" period (period during which hMG was not given, ie the interval between the last hMG dose to the hCG injection, in hours) of at least 32 hours was allowed (Brown, 1978).

#### EGG RECOVERY

Laparoscopy was done 24-26 hours after the onset of the LH surge, or 34-36 hours after the hCG injection. The technique of oocyte aspiration was that described by Renou, Trounson, Wood et al (1981), using a single lumen needle (Monash oocyte aspiration

needle, Rocket of London).

In phase I, there were 22 laparoscopic egg recoveries (table 3.1). The majority of cases that did not reach laparoscopy had abnormal follicular growth (see chapter 5). Of the 22 cases that had laparoscopic egg recovery, 14 had spontaneous LH surge; 12 oocytes were recovered from 11 patients (table 3.2).

In phase II, there were 100 patients in 105 cycles with 100 laparoscopies and 238 eggs were recovered from 85 patients (table 3.2).

#### FERTILIZATION AND GROWTH IN VITRO

After the oocyte was collected, it was matured for 6 hours in Whittingham's T6 medium before insemination (Ng, Ratnam, Law et al, 1985b). The semen was collected about an hour before insemination, and washed twice with T6 and centrifuged at 1800 rpm before layering for half an hour at 37°C. Spermatozoal concentrations of 0.25 to 0.50 million/ml were used. The egg was examined 16-18 hours later for evidence of fertilization, viz. two pronuclei and two or more polar bodies. Twenty-four hours later, the zygote reached the 2-4 cell stage. The medium for fertiliza-

tion and growth was T6 supplemented with heat inactivated homologous human serum at 10% and 15% (v:v) respectively.

#### EMBRYO REPLACEMENT

Replacement of the pre-implantation embryo was between the 2- to 8-cell stage. The technique was as described by Leeton, Trounson, Jessup et al (1982). Briefly, the embryos were drawn into an internal catheter together with 30 - 50 ul of transfer medium using a 1 ml tuberculin syringe. An external sleeve was then introduced gently without anaesthesia through the cervical canal just past the internal os. The internal catheter was then pushed through into the uterine cavity through the external sleeve such that it protuded 2 - 4 cm beyond the tip of the sleeve. The embryos were then displaced into the uterine cavity by depressing the plunger of the attached syringe.

#### LUTEAL PHASE SUPPORT

In phase I, the luteal phase was supported with 500 mg of 17OH-progesterone caproate (Depo Proluton, Schering AG) injected twice a week.



In phase II, the luteal phase was supported by either progesterone or a placebo (vit B Co) in a randomised trial (see chapter 10 for details). Plasma was collected from the patients once every 2 days from the day of oocyte recovery and assayed for  $E_2$ , P and beta-subunit of hCG. Plasma P and hCG were assayed retrospectively.

### PREGNANCIES

In phase I there was one pregnancy in 8 replacements (12.5%). In phase II there were 11 pregnancies in 59 replacements (18.6%); 2 were biochemical pregnancies while 4 were abortions and 5 were singleton pregnancies that delivered normal children at term. When the number of embryos replaced were considered (see table 3.3), the rates improved with multiple embryo replacements, up to 3 embryos.

### RADIOIMMUNOASSAY (RIA) OF ESTRADIOL-17B AND PROGESTERONE

Estradiol-17B ( $E_2$ ) and progesterone were measured by specific RIA with reagents supplied by WHO (WHO Method Manual, 6th edition,

1982). Specific antisera to P and E<sub>2</sub> were raised in rabbits by coupling 3-carboxymethyl oxime-bovine serum albumin with the respective steroid. For the E<sub>2</sub> assays, 500 ul of plasma was extracted with freshly distilled diethylether. The organic ether phase was pipetted and dried by heating in a water bath at 45°C. The extracts were dissolved in 0.5 ml of phosphate buffered saline at pH 7.2 containing 0.1% gelatin (assay buffer). Standards made up in assay buffer with pure E<sub>2</sub> at a range between 7.8 to 250 pg/ml were analysed with each set of samples assayed. Duplicate aliquots (500 ul) were placed in tubes for RIA. For the zero blank (B<sub>0</sub>) representing maximum binding of antibody and tracer, 500 ul of assay buffer was added. Tubes for nonspecific binding and total counts were added with 600 ul buffer. 100 ul aliquots of anti-E<sub>2</sub> was added to all tubes except total count and non-specific binding tubes and incubated for 1/2 hour at room temperature. This was followed by the addition of 100 ul of <sup>3</sup>H-E<sub>2</sub> to all tubes and incubation for 1 hour at 4°C. 200 ul of ice-cold dextran-coated charcoal (0.625 g charcoal Norit A with 0.625 g dextran in 100 ml assay buffer) was then added and vortex mixed. Thereafter all tubes were reincubated for 15 min at 4°C. At the end of the incubation period, the tubes were centrifuged at 2300g for 5 min at 4°C. The supernatant was decanted into a scintillation vial containing 10 ml of toluene-based scintillation fluid with Triton X-10. Radioactivity was estimated in a Hewlett-

Packard liquid scintillation spectrometer (Packard Tri-Carb 300) for 5 min having 65% efficiency for tritium.

P assays were performed with specific antisera raised in rabbits by the same method described above except that the incubation period after addition of the radiolabelled tracer, antiserum and standards (range between 12 and 200 ng/ml) or samples was 24 hours at 4°C. The intra- and inter- assay coefficient of variation for E<sub>2</sub> was 7.3% and 10.4% respectively while that for P was 6.7% and 9.6% respectively.



TABLE 3.1: INDICATIONS FOR INCLUDING PATIENTS IN IVF PROGRAM

Indications	Phase I (July 82-May 83)	Phase II (Sept 83-Sept 85)
-----	-----	-----
Tubal obstruction	13	48
Tubal disease	-	5
Sperm problems	4	9
Endometriosis	1	6
Idiopathic	4	8
Immunological	-	2
Combination	-	22
	-----	-----
Total	22	100

TABLE 3.2: OUTCOME OF LAPAROSCOPIC EGG RECOVERIES

	Phase I	Phase II
	-----	-----
Laparoscopies	22	100
Successful recoveries	11	85
Oocytes recovered	12	238
Fert.& cleavage	9	116
Embryo replacements	8	59
Pregnancies	1	11

TABLE 3.3: RELATIONSHIP OF NUMBER OF EMBRYOS REPLACED TO PREG-  
NANCY RATES

Number embryos replaced	Phase I		Phase II	
	Cases	Pregn	Cases	Pregn
1	7	1 (14.3%)	15	2 (13.3%)
2	1	-	25	5 (20.0%)
3			14	4 (28.6%)
4			3	-
5			2	-
Total	8	1 (12.5%)	59	11 (18.6%)



## CHAPTER 4:

### SINGAPORE'S FIRST TEST-TUBE BABY

#### SYNOPSIS

The fourth patient in the IVF program was a 25 year old Chinese female with 4 years of primary subfertility. In her August cycle, she was on clomiphene citrate 50mg tds from day 2 to day 6. By day 12 she had a single follicle on her right ovary measuring 1.4 \* 1.6 \* 1.6 cm and was admitted. On day 15 the follicle was 1.9 \* 2.5 \* 1.8 cm, with a cumulus oophorus measuring 0.7 cm. As 3 hourly urine for LH showed no spontaneous surge, hCG 5000 i.u. was given and the laparoscopy was performed 30 hours later. A single follicle on the right ovary, measuring 2.5 cm in diameter, was aspirated. A single preovulatory oocyte was obtained and cultured in T6 medium for 6 hours before insemination. Seventeen hours later 2 pronuclei were noted. The 4-cell preimplantation embryo was transferred 46.5 hours after insemination. On the 37th day after laparoscopy, a gestational sac was seen on ultra-sound. The patient delivered on 19th May 1983 after induction.

## INTRODUCTION

The embryo culture laboratory was started in Singapore in December 1981 after gaining experiences from Monash University, Melbourne, over a period of 4 weeks in June 1981. The initial 6 months were occupied with murine experiments. Only after it was possible to obtain cleavage in 80 - 100% of murine eggs fertilized in vitro to 2-cell stages that the IVF program on the human commenced (July 1982).

The first patient was a 29 year old Chinese who had bilateral tubal damage. She had 3 follicles after clomiphene stimulation. Laparoscopic recovery of eggs was unsuccessful, possibly because two Falcon test-tubes had to be rotated throughout the operation. The second patient was a case of irreversible tubal occlusion, and had only one follicle following clomiphene. Her laparoscopy was timed 25 hours from the onset of the spontaneous LH surge; however, the laparoscopy was too late as ovulation had occurred spontaneously. The third patient was a 30 year old Indian who had a previous salpingectomy for an ectopic pregnancy, and the remaining tube was hypoplastic. An egg was recovered from a single follicle following clomiphene, and after fertilization developed to 8-cells in 59 hours. However, the embryo transfer was difficult and no pregnancy ensued.

## THE PATIENT

The fourth patient resulted in our first IVF pregnancy. She was a 25 year old Chinese who had 4 years of primary subfertility due to bilateral cornual obstruction of her Fallopian tubes. Her last menstrual period was on 28 August 1982. Clomiphene was taken at 50 mg t.d.s. from day 2 for 5 days. On her 10th day, ultrasound examination revealed a follicle  $1.1 * 1.1 * 1.1$  cm on her right ovary. This grew to  $1.4 * 1.6 * 1.6$  cm on day 12 when she was admitted, and to  $1.9 * 2.5 * 1.8$  cm on day 15 with a cumulus oophorus measuring 0.7 cm. Her serum  $E_2$  measured 232 pg/ml on day 10, and increased to 1085 pg/ml on day 14 (see table 4.1). Three-hourly urine measurements for LH by Higonavis from day 12 showed no spontaneous surge. Intra-muscular hCG 5000 i.u. was given on day 15 and the laparoscopic recovery timed 30 hours later. A single pre-ovulatory follicle measuring 2.5 cm in diameter was seen and aspirated and a preovulatory egg was obtained.

## FERTILIZATION AND GROWTH

The egg was matured for a further 6 hours in 0.5ml of T6 medium



with 10% heat inactivated human serum and 0.5ml of follicular fluid before insemination. The semen was collected 2 hours before insemination, washed with medium, centrifuged at 500 rpm for 10 minutes and layered with 0.5ml medium. After 30 minutes the supernatant was collected. Half a million treated spermatozoa in 27 ul were then introduced into the Falcon tube with the ovum. After 17.5 hours, 2 pronuclei were seen. Growth times were within those reported by Edwards et al, 1981 (see table 4.2).

#### EMBRYO REPLACEMENT

The 4-cell embryo was transferred using the method described by Leeton, Trounson, Jessup et al, 1982. The catheter used was a Monash Embryo Transfer Set I (William A. Cook Australia Pty Ltd.) The transfer was carried out with the patient in lithotomy position without any anaesthesia and any difficulty. The volume of medium transferred with the embryo was 20 ul.

#### POST REPLACEMENT REGIME

The patient was placed on complete rest in bed in the supine position for 24 hours. Depo Proluton (17-OH progesterone

caproate, Schering AG) was administered at a dosage of 250 mg on POD1 (1st day after laparoscopy), 500 mg on POD2, POD3, POD5 and subsequently twice a week from POD12. Her B-hCG showed a rise by the 11th POD (9th day after ER) (see table 4.3). Ultra-sound examination showed a gestational sac on the 23rd POD, measuring 5 \* 5 \* 4 mm.

Her pregnancy progressed satisfactorily. Ultra-sound biparietal diameters were within acceptable limits (see figure 1), while 24 hour urinary estriols were satisfactory. Antenatal cardiotocographs from the 32 week of gestation were reactive. Labour was surgically induced on 19th May 1983, at the beginning of the 38th week of gestation and was delivered by forceps 7.5 hours later. The healthy male infant, weighing 2535 gm, was without any obvious congenital defects.

TABLE 4.1: FOLLICULAR VOLUME AND SERUM ESTRADIOL LEVELS IN FIRST PREGNANCY

Day of cycle	Serum estradiol (pg/ml)	Follicular volume (cc)
-----	-----	-----
10	232	0.70
11	408	1.33
12	396	1.88
13	795	2.27
14	1085	2.67
15	1152	4.48
16	1073	2.26



TABLE 4.2: IN VITRO GROWTH OF EMBRYO IN FIRST PREGNANCY

Stage	Time from insemination (hours)	Expected time *
-----	-----	-----
2 pronuclei	17.5	-
3 blastomeres	34.75	-
4 blastomeres	46.0	49.0 ± 1.3

\* Ref: Edwards, Purdy, Steptoe et al, 1981.

TABLE 4.3: ESTRADIOL, PROGESTERONE AND HUMAN CHORIONIC  
GONADOTROPIN LEVELS FOLLOWING LAPAROSCOPY

Days after laparoscopy	Estradiol (pg/ml)	Progesterone (ng/ml)	Human chorionic gonadotrophin (miu/ml)
-----	-----	-----	-----
5	387	23.6	10.3
8	281	21.0	4.9
11	416	28.9	>500
14	804	36.9	>500
17	518	30.8	>500
20	935	41.2	>500

## CHAPTER 5:

### FOLLICULAR PHASE IN UNSTIMULATED AND CLOMIPHENE-INDUCED CYCLES

#### SYNOPSIS

Patients were initially either unstimulated or stimulated with clomiphene citrate 50 mg tds for 5 days from day 2. Previous studies of  $E_2$  response assume that eggs were present in the follicles monitored. Only patients in whom at least one egg was recovered at laparoscopy were considered in this analysis. Six patients had clomiphene and 4 patients were unstimulated. One to 3 follicles were present in the clomiphene-treated group, while all the unstimulated patients had 1 follicle each. Four of the clomiphene-induced patients required hCG administration, as opposed to 1 patient in the unstimulated group while the rest had spontaneous LH surge. The mean plasma  $E_2$  level varied from 195 pg/ml/follicle 7 days before laparoscopy to 633 pg/ml/follicle on the day of the laparoscopy in the clomiphene-treated patients, and from 87 pg/ml/follicle 7 days before laparoscopy to 473 pg/ml/follicle on the day of the laparoscopy in the unstimulated group. In 5 of the 10 patients, the  $E_2$  levels fell on the day of laparoscopy, with the maximal values on the day prior to the



recovery. Only 2 of the 6 clomiphene-treated patients showed a continued rise.

INTRODUCTION

Initial attempts were with unstimulated cycles in order to mimic the natural state. hMG was used subsequently in an attempt to improve control of the cycles but was unsuccessful (Steptoe and Edwards, 1979). This was later attributed to the use of a hMG regime designed for anovulatory cycles (Edwards, 1983). The first two babies delivered after IVF and ER were from unstimulated cycles (Edwards, Steptoe and Purdy, 1980; Lopata, Johnston, Hoult et al, 1980).

In 1981 Trounson, Leeton, Wood et al reported the successful use of clomiphene citrate in stimulating multiple follicular growth for IVF and ER, although its use had been advocated by Lopata, Brown, Leeton et al in 1978. This has led to its widespread use by many IVF and ER centres. More recently, hMG has been used successfully for IVF & ER (Jones, Jones, Andrews et al, 1982; Wortham, Veeck, Witmyer et al, 1983) and this drug is now routinely used.

Phase I involved clomiphene citrate-induced and unstimulated cycles. The purpose of this chapter is to compare the  $E_2$  response in these two regimes.

## MATERIALS

The data presented were confined to subjects where at least one egg was recovered at laparoscopy. This was to exclude the possibility of abnormal follicular development and "empty" follicles (Coulam, Bustillo and Schulman, 1986). This is in contrast with most clinical studies where ovulation is supposed, but not definitely proven, to have occurred.

In phase I, there were 22 patients involved in 33 patient cycles. There were 22 laparoscopic egg recoveries and the majority of cases that did not reach laparoscopy had abnormal follicular growth (table 5.1). Twelve eggs were recovered from 11 patients. Of these 11 patients, one received hMG and this patient was excluded. Thus, for this analysis, there were 10 patients (6 clomiphene-induced and 4 unstimulated), ranging in age from 25 to 39 (mean 31.9 years). Nine patients had tubal obstruction, and one patient had idiopathic subfertility.

The treatment regime, egg collection, in vitro fertilization and growth, and embryo replacement were as described in Chapter 3 in the Materials and Methodology section.

## RESULTS

The daily plasma  $E_2$  levels are given in table 5.2. Only 1 patient in the clomiphene-induced cycles had 1 follicle seen on laparoscopy, whilst all patients in the unstimulated cycles had 1 follicle each. Four of 6 patients in the clomiphene-induced cycles needed hCG injection, as opposed to 1 out of 4 in the unstimulated cycles. The plasma  $E_2$  levels per follicle in the clomiphene-induced group are given in table 5.3. The range varied from 358 to 1255 pg/ml. The mean levels per follicle are given in table 5.4 and these were higher in the clomiphene-induced cycles.

## DISCUSSION

Edwards, Steptoe and Purdy (1980) suggested that ovarian stimulation with hMG may disturb the menstrual cycle, inducing a shorter luteal phase and a disorganised endometrium, both incompatible with establishing a pregnancy. It was subsequently shown



(Trounson, Leeton, Wood et al, 1981; Johnston, Lopata, Speirs et al, 1981) that cycles induced by clomiphene citrate and hMG for IVF & ER were compatible with pregnancies. Stimulated cycles resulted in more preovulatory follicles, more eggs at laparoscopy, and higher pregnancy rates (Trounson and Conti, 1982).

In unstimulated cycles in normal Chinese women, the peak level of total oestrogen was 508.8 pg/ml (Salmon, Chew and Ratnam, 1976). Saxena, Dusitsin and Vichai (1974) showed that the hormone profiles for estrogen, LH and P were similar in both Caucasian and Thai women. In our group of unstimulated cycles, a mean peak value of plasma  $E_2$  of 473 pg/ml was observed on the day of oocyte recovery. This is compatible with the level of  $E_2$  (500 pg/ml) associated with successful IVF & ER cycles (Trounson, 1982).

In clomiphene-induced cycles, the mean  $E_2$  levels observed per follicle were much higher with values of 700 pg/ml on the day prior to and 633 pg/ml on the day of the laparoscopy. It is possible that clomiphene induced the growth of several follicles of which only a few ovulate and that the remainder do not continue to the preovulatory stage, but contribute to steroidogenesis. It was also observed by Smith, Picker, Sinosich et al (1980) that the mean plasma  $E_2$  levels in clomiphene-induced cycles were sig-

nificantly higher than natural cycles, even when only one follicle was present.

In general, a rising trend in the  $E_2$  levels was observed with follicle maturation. The large standard deviations noted in this study were partly due to the small sample sizes. On the day of egg recovery, the range of plasma  $E_2$  per follicle varied from 358 to 1255 pg/ml in clomiphene-induced cycles, and from 390 to 629 pg/ml in unstimulated cycles. Absolute  $E_2$  levels are therefore poor indicators of follicular maturity. Rising trends were more reliable indicators of continued follicular development. Such a trend may lead to an endogenous LH surge before hCG could be administered; hence an accurate formula to predict when the LH surge could occur would be useful clinically (see chapter 9). In fact stimulated regimes should aim at a high proportion of spontaneous LH surges; this would be an index of satisfactory endocrinological control in spite of an increase in the number of follicles.

In 5 of the 10 patients, the  $E_2$  levels fell on the day of laparoscopy, the maximal values having occurred one day prior to the recovery. For an IVF program, this pattern is less satisfactory than if the  $E_2$  levels had continued to rise after spontaneous LH surge or after injection of hCG. Pregnancy rates

were better in those in whom  $E_2$  levels continue to rise (Jones, Acosta, Andrews et al, 1983). It is of interest to note that of the 6 clomiphene-induced cycles, only 2 showed a continued  $E_2$  rise. This compared unfavourably with unstimulated cycles where in 3 of 4 subjects the  $E_2$  levels continued to rise.



TABLE 5.1: CASES THAT DID NOT PROCEED TO LAPAROSCOPY

	Abn.foll.growth	Others	Total
	-----	-----	-----
Clomiphene	5	4 *	9
Unstimulated	1	1 **	2
	-----	-----	---
	6	5	11

Note: \* 1 trial, 1 flu, 1 matrimonial disharmony, 1 husband with rubella

\*\* Dense shadow in endometrium on u/s ?tb

TABLE 5.2: DAILY PLASMA ESTRADIOL (pg/ml) LEVELS PRIOR TO OOCYTE

RECOVERY

Days prior to oocyte recovery								hCG	Number of	
-----								(iu)	-----	-----
7	6	5	4	3	2	1	0	----	foll.	oocytes
-	-	-	-	-	-	-	-		-----	-----

Clomiphene-induced cycles:

-----											
1.	-	-	-	337	207	189	766	1014	5000	2	1
									D14		D15
2.	-	-	640	880	1892	2067	2119	1614	5000	2	1
									D11		D12
3.	-	451	-	672	644	871	694	971	-	2	1
									5000		D13
4.	195	320	399	478	331	638	1045	763	5000	2	2
									D13		D14
5.	-	-	-	505	-	978	1500	1255	-	1	1
											D13
6.	-	232	408	396	795	1085	1152	1073	5000	3	1
									D15		D16

TABLE 5.2: DAILY PLASMA ESTRADIOL (pg/ml) LEVELS PRIOR TO OOCYTE RECOVERY (continue)

Days prior to oocyte recovery								hCG	Number of	
-----								(iu)	-----	
7	6	5	4	3	2	1	0	----	foll.	oocytes
-	-	-	-	-	-	-	-	-	-----	-----
Unstimulated cycles:										
-----										
7.	-	-	190	229	381	453	588	629	-	1
										1
										D16
8.	-	-	117	-	-	475	645	422	-	1
										1
										D12
9.	87	87	56	128	132	176	229	451	-	1
										1
										D16
10.	-	123	-	-	139	267	388	390	5000	1
										1
									D12	D13



TABLE 5.3: PLASMA ESTRADIOL LEVELS (pg/ml) PER FOLLICLE IN

CLOMIPHENE-INDUCED CYCLES

Patient	Day prior to oocyte recovery							
	7	6	5	4	3	2	1	0
1.	-	-	-	169	104	95	383	507
2.	-	-	320	440	946	1034	1060	807
3.	-	226	-	336	322	436	347	486
4.	195	160	200	239	166	319	523	382
5.	-	-	-	505	-	978	1500	1255
6.	-	77	136	132	265	362	384	358

TABLE 5.4: MEAN ESTRADIOL VALUES (pg/ml) PER FOLLICLE IN CLOMIPHENE-INDUCED AND UNSTIMULATED CYCLES

	Day prior to oocyte recovery							
	7	6	5	4	3	2	1	0
	-	-	-	-	-	-	-	-
Clomiphene-induced cycles:								
Mean values	195	154	219	304	361	537	700	633
Std.deviation (+/-)				150	338	381	474	344
Unstimulated cycles:								
Mean values	87	105	121	179	217	342	463	473
Std.deviation (+/-)				72	142	145	191	107

## CHAPTER 6:

### FOLLICULAR PHASE IN CLOMIPHENE-HUMAN MENOPAUSAL GONADOTROPIN CYCLES

#### SYNOPSIS

Two clomiphene-hMG regimes were compared. Clomiphene 50mg b.d. was administered from day 2 for 5 days while hMG was given from day 6. In the first regime 1 ampoule (75 iu) per day was given for the first 3 days, and in the second, 2 ampoules (150 iu) per day. The subsequent dosages were dependent on the  $E_2$  response. There were 9 cases for the first regime, and 10 cases for the second. The mean number of hMG ampoules given were 16.50 and 19.25 respectively. The follicles seen on ultrasound were  $3.0 \pm 0.5$  and  $3.4 \pm 1.2$  respectively. There was no statistical difference in the  $E_2$  response up to the day of laparoscopic egg recovery for the two regimes. However, spontaneous LH surge was observed in 4 of 9 cases in the first group and 6 of 10 cases in the second group. When a comparison was made between cases that had a spontaneous LH surge and cases that were given hCG there was a higher  $E_2$  level on the day of the laparoscopy in the hCG



group with the lower hMG regime ( $p < 0.05$ ). There were no other differences.

A 52.6% incidence of spontaneous LH surge with clomiphene-hMG was observed. Hence such stimulated regimes can result in a high proportion of spontaneous LH surges. This may be an index of satisfactory endocrinological control in spite of an increase in the number of follicles.

## INTRODUCTION

The hMG regime was first used for IVF & ER by Steptoe and Edwards (1979), but was successfully implemented only in 1981 (Johnston, Lopata, Speirs et al, 1981; Jones, Jones, Andrews et al, 1982). Since then, there are many hMG regimes used all over the world. The use of hMG induces multiple follicular development and hence more eggs may be recovered at laparoscopy. A higher number of embryos replaced results in a higher pregnancy rate (Trounson and Wood, 1981; Biggers, 1981; Muasher, Wilkes, Garcia et al, 1984). However, fears have been expressed of shortened luteal phase (Johansson and Gemzell, 1969) and increased abortion rates (Edwards and Steptoe, 1983) when the  $E_2$  levels in the follicular phase become too high. This is particularly so when

hMG is used (Olson, Rebar, Schreiber et al, 1983).

A combination of an anti-estrogen such as clomiphene citrate with hMG has also been used with some success (Seppala, 1985). Laufer, de Cherney, Haseltine and co-workers (1983) believed that clomiphene may have negative effects on the ovum and corpus luteum. However, clomiphene has been used very successfully in IVF (Trounson, Leeton, Wood et al, 1981; Edwards and Steptoe, 1983). Moreover, it is possible that clomiphene in combination with hMG may protect against shortened luteal phase seen in hMG-induced cycles (Edwards, 1985).

It is preferable for  $E_2$  in the follicular phase to approach non-stimulatory levels (Jones, Acosta, Andrews et al, 1983). When the clomiphene-hMG regime in KK Hospital was started, it was to determine whether lower doses of hMG in the initial stages resulted in such an  $E_2$  response. The Norfolk group has used hMG at 2 or 3 ampoules a day for the first 2 days of treatment and found that the higher dose resulted in more preovulatory oocytes but lower pregnancy rates (Jones, 1984). This chapter presents experiences with either 1 or 2 ampoules of hMG in the first 3 days of hMG administration.

## MATERIALS AND METHODS

The clomiphene-hMG regime started in September 1983 (phase II). The stimulation regime and monitoring were described in Chapter 3.

The first 10 consecutive cases were assigned to regime I (lower hMG dose), and the next 10 cases to regime II (higher hMG dose). Unfortunately one 40 year old patient in the first group had abnormal follicular development and was excluded. She also had similar follicular phases in 2 previous cycles, one with clomiphene alone and another unstimulated. The patients' ages ranged from 29 to 40 years old (mean: 33.6 years) in the first group, and from 29 to 36 (mean: 33.0 years) in the second. Their indications for IVF & ER were tubal (7 in each group), tubal and oligospermia (2 in the second group), and prolonged idiopathic subfertility (2 and 1 in the first and second groups respectively).

Statistical analysis was done using the Student's t-test.

## RESULTS



The mean number of hMG ampoules given in regime I was 16.5 (range 4 - 29) and 19.25 in regime II (range 13 - 31). The mean number of follicles seen ultrasonically on day 9 was 3.0 in regime I (range 2 - 4) and 3.4 in regime II (range 1 - 5). The ultrasonic observation of follicle number was used here because pelvic adhesions prevented the determination of follicle numbers laparoscopically in 3 patients in the first group and 6 patients in the second group. The mean day of laparoscopy was on 14.6 for regime I (range 12 - 16) and 13.4 for regime II (range 12 - 15). There were no statistical differences between hMG dosages, number of follicles observed on ultrasound, and the day of laparoscopy between the two regimes.

The mean plasma  $E_2$  values are given in table 6.1. The values between the two regimes were not significantly different,  $p > 0.05$  (figure 6.1).

Spontaneous LH surge occurred in 4 of 9 patients in the first group, and in 6 of 10 patients in the second group. When the results were analysed according to whether spontaneous LH surge occurred or hCG was given, there was no significant difference between the  $E_2$  levels in the 2 regimes (figures 6.2 and 6.3), except for the value on the day of laparoscopic recovery in regime I (6.2). The mean  $E_2$  level on that day was  $606.9 \pm 254.9$  ( $M \pm$

SD) pg/ml/foll for the group where hCG was given, as opposed to 270.6  $\pm$  41.6 pg/ml/foll for the group with spontaneous LH surge ( $p < 0.05$ ). There was also no significant difference in the hMG dosage, number of follicles observed and the day of laparoscopy between the 2 groups in each of the two regimes.

In spontaneous LH cycles more hMG was required, resulting in more follicles but lower levels of  $E_2$  per follicle. However, these observations were not statistically significant ( $p > 0.05$ ).

Laparoscopy resulted in 9 eggs from 6 patients from regime I, and 14 eggs from 6 patients from regime II. Those unsuccessful cases had severe pelvic adhesions. Fertilization and cleavage were seen in 5 eggs in the first group, and 9 eggs in the second. Embryo replacement was carried out in 4 patients from regime I, and 5 patients from regime II. One pregnancy resulted from regime II, and has since delivered a normal term infant.

## DISCUSSION

There was no statistical difference between the 2 regimes. In fact, despite increasing the FSH dosage during days 6 to 9 (regime II), a higher mean total hMG dose was required.

The difference in the  $E_2$  levels on the day of laparoscopic egg recovery between the spontaneous LH group and hCG group noted in regime I was not observed in regime II. This observation may therefore not be important since a bigger series may not show this difference.

For an IVF program, pregnancy rates appear to be better if  $E_2$  levels continue to rise after a spontaneous LH surge or after injection of hCG (Jones, Acosta, Andrews et al, 1983). Unstimulated cycles are more likely to show a continued  $E_2$  rise than clomiphene-induced cycles (Ng, Ratnam, Yeoh et al, 1985). For the two clomiphene-hMG regimes, the mean  $E_2$  level fell after spontaneous LH or hCG injection.

The literature suggests that it is not often that a spontaneous LH surge occurs in a cycle treated with hMG alone (Ferraretti, Garcia, Acosta et al, 1983; Laufer, De Cherney, Haseltine et al, 1983). In fact there was no LH surge noted in 191 cycles treated in the Norfolk program (Ferraretti, Garcia, Acosta et al, 1983). However, in IVF cycles treated with clomiphene-hMG there is a variable degree of spontaneous LH surge (Trounson and Wood, 1984). We had a high incidence of spontaneous LH surge, 4 of 9 cycles from regime I and 6 of 10 from regime II. The timing of



hCG administration affected the frequency of spontaneous LH surges, and inversely the day of laparoscopic recovery. The effects of shortening the follicular phase by administering the hCG injection earlier on the quality of the oocytes recovered were discussed in chapter 7.

Edwards and Steptoe (1983) reported that clomiphene treated cycles with spontaneous LH surges were better than clomiphene treated cycles with hCG for IVF successes. They suggested that the LH surge was more "physiological" than the hCG injection and this resulted in embryos or uteri superior for implantation. However, with their clomiphene-hMG series, the data suggested that the better embryos were from patients given hCG, and not those with spontaneous LH surge (Edwards, 1985). On the other hand, Trounson and Wood (1984), who had been using clomiphene with hMG since 1981, had an incidence of 62.0% spontaneous LH surge with the 305 patients so treated, and 63.8% of their embryo replacements and 76.7% of their pregnancies came from this group.

Our series showed a 52.6% incidence of spontaneous LH surge with clomiphene-hMG. There was one pregnancy in 5 replacements in regime II, but no pregnancy in 4 replacements in regime I. After completion of this study we continued with regime II because of higher number of follicles and earlier laparoscopic recovery day.

Such stimulated regimes can result in a high proportion of spontaneous LH surges with resulting pregnancies. This may be an index of satisfactory endocrinological control in spite of an increase in the number of follicles.

However, the presence of spontaneous LH surge results in logistic difficulties in many IVF programmes. This is due mainly to the short notice for egg recovery after the detection of the onset of the LH surge. Many IVF programmes cancel such cases. In fact, there is now a tendency to suppress the spontaneous LH surge by down-regulation of the pituitary with analogues or antagonists of gonadotropin releasing hormone (Sharma, Williams, Whitehead et al, 1986). This is further discussed in chapter 8.

It is possible that clomiphene-hMG cycles for IVF offer advantages over hMG cycles without clomiphene. Firstly, there is an increased likelihood of spontaneous LH surge. Logistically, this may not be an advantage, but it is likely to be more physiological. Secondly, the anti-estrogenic effect of clomiphene may protect the corpus luteum from early luteolysis (Trounson and Leeton, 1982), thus decreasing the possibility of a short luteal phase. Thirdly, clomiphene may also protect the endometrium from the high estrogenic levels reached with such stimulation. Excessive estrogenic stimulation results in abnormal endometrial

growth (Novak and Woodruff, 1979). Clomiphene binds to estrogen receptors in the female genital tract and the receptor complex is retained in the nucleus for long periods (Edwards, 1980). Fourthly, there is a possiblity of better pregnancy rates (Trounson and Wood, 1984)). This needs further verification as there seems to be conflicting data by Edwards (1985). Fifthly, there is no need for hMG administration in the early follicular phase, and this is an advantage when patients come from overseas for treatment.



TABLE 6.1: MEAN PLASMA ESTRADIOL LEVELS (pg/ml) PER FOLLICLE IN  
CLOMIPHENE-HUMAN MENOPAUSAL GONADOTROPIN CYCLES

hMG. Foll.		Day prior to oocyte recovery								
---	----	-----								
(amp)		8	7	6	5	4	3	2	1	0
		-	-	-	-	-	-	-	-	-
Regime I:										
-----										
M	6.5 3.0	84.0	153.6	222.8	225.4	303.4	397.9	595.2	589.9	457.4
SD	7.0 0.5	10.9	34.8	72.3	50.6	128.0	139.9	255.4	243.2	254.1
n	9 9	4	6	7	8	8	9	9	9	9
Regime II:										
-----										
M	19.25 3.4		113.9	135.5	179.4	257.3	340.0	534.9	661.8	479.5
SD	6.0 1.2		41.9	63.2	75.8	101.9	126.9	174.1	208.6	181.6
n	10 10		2	4	8	10	10	10	10	9

FIGURE 6.1: PLASMA ESTRADIOL LEVELS FOR CLOMIPHENE-HUMAN  
MENOPAUSAL GONADOTROPIN REGIMES

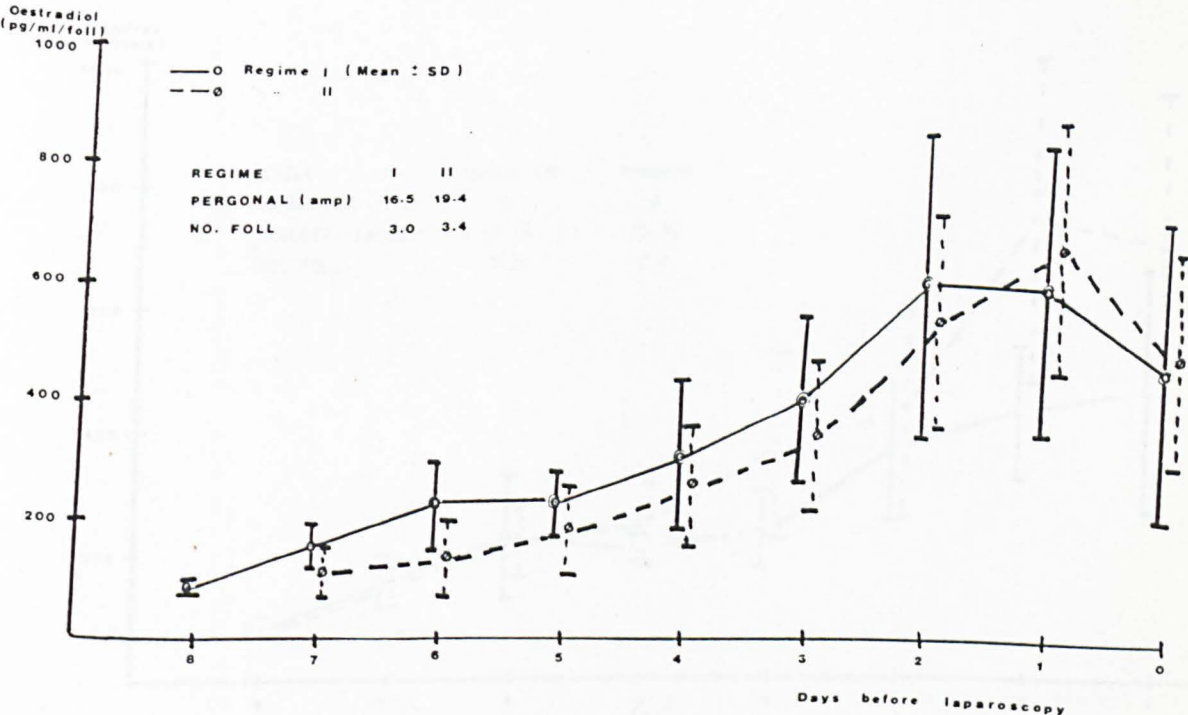


FIGURE 6.2: PLASMA ESTRADIOL LEVELS FOR CLOMIPHENE-HUMAN  
 MENOPAUSAL GONADOTROPIN REGIME I - SPONTANEOUS L.H. SURGE  
 COMPARED TO H.C.G. ADMINISTRATION

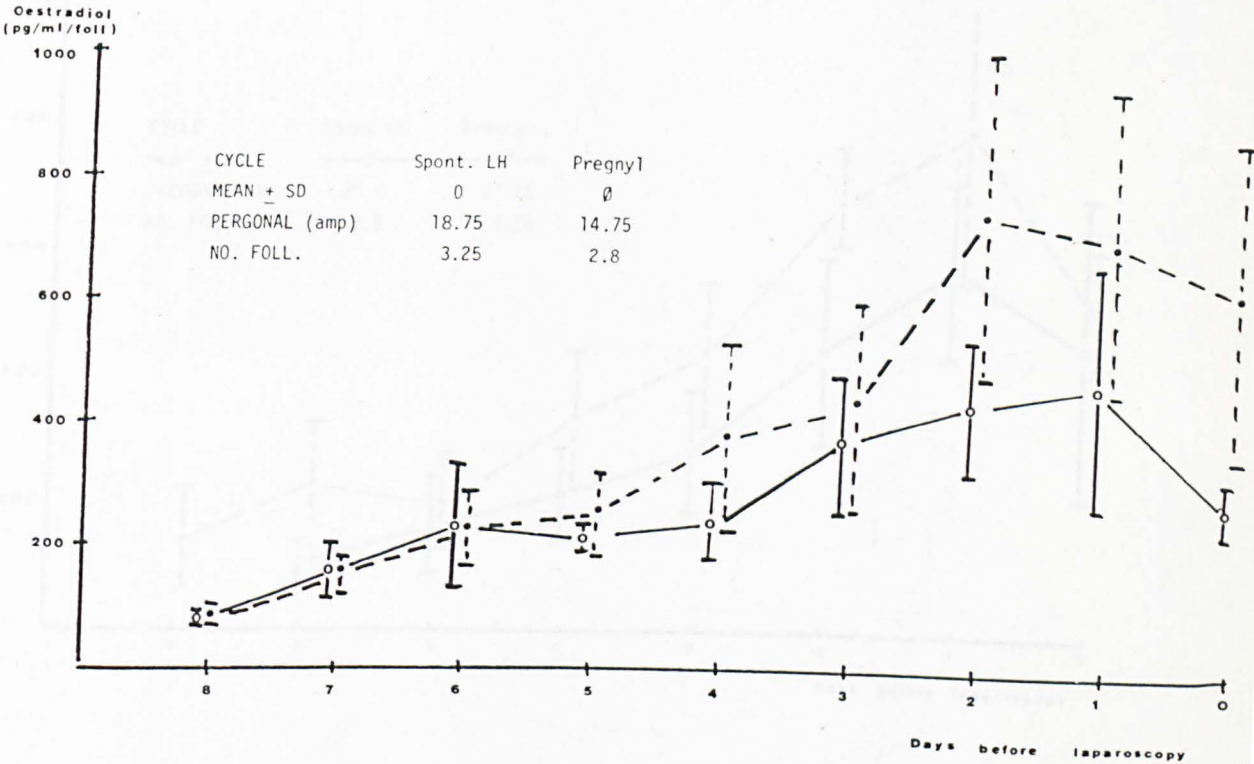
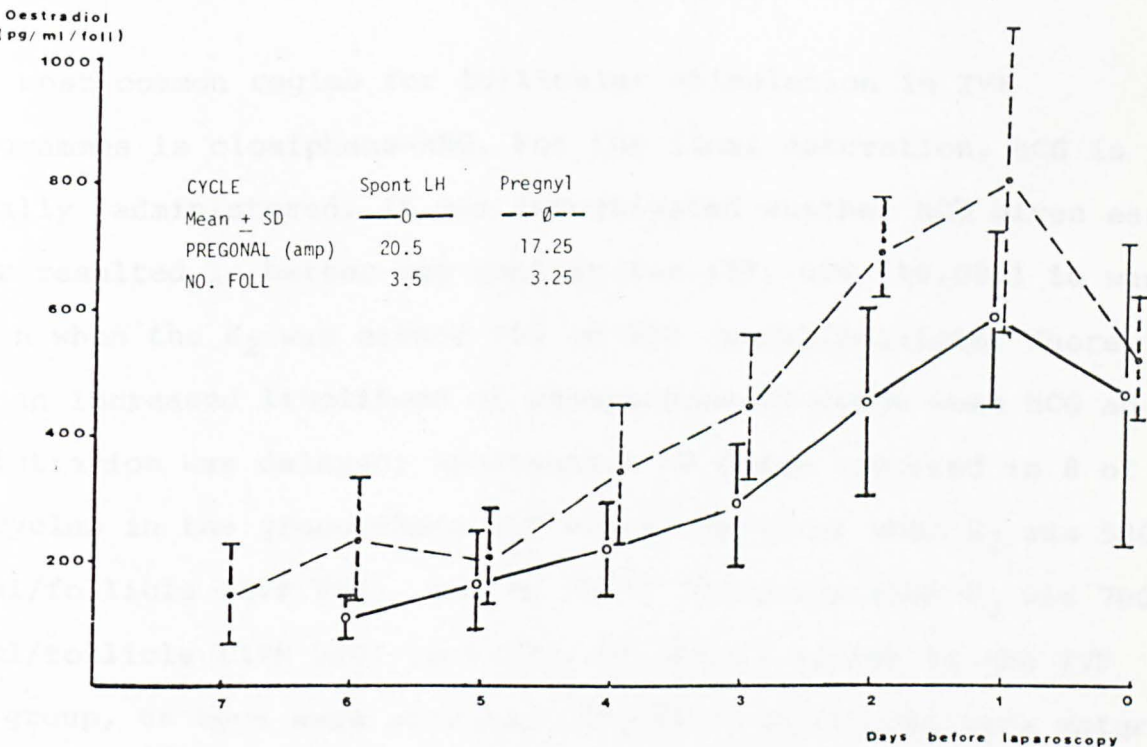




FIGURE 6.3: PLASMA ESTRADIOL LEVELS FOR CLOMIPHENE-HUMAN  
 MENOPAUSAL GONADOTROPIN REGIME II - SPONTANEOUS L.H. SURGE  
 COMPARED TO H.C.G. ADMINISTRATION



## CHAPTER 7

### EARLIER ADMINISTRATION OF HUMAN CHORIONIC GONADOTROPIN

#### SYNOPSIS

The most common regime for follicular stimulation in IVF programmes is clomiphene-hMG. For the final maturation, hCG is usually administered. It was investigated whether hCG given earlier resulted in better egg quality for IVF. hCG (10,000) iu was given when the  $E_2$  was either 700 or 500 pg/ml/follicle. There was an increased likelihood of spontaneous LH surge when hCG administration was delayed; spontaneous LH surge occurred in 8 of 27 cycles in the group where hCG was to be given when  $E_2$  was 500 pg/ml/follicle (IVF 500), and in 14 of 22 cycles when  $E_2$  was 700 pg/ml/follicle (IVF 700) ( $p < 0.05$ ). Of the 22 cycles in the IVF 700 group, 66 eggs were obtained. Thirty-five (53.0%) were mature and 26 (39.4%) were intermediate, as judged morphologically. Of the 27 cycles in the IVF 500 group, 92 eggs were obtained; 55 (59.8%) were mature and 36 (39.1%) intermediate. Hence there were more preovulatory eggs (mature + intermediate) when hCG was given earlier (92.4% for the group where hCG was given at 700

pg/ml/follicle, compared with 98.9% for the 500 pg/ml/follicle group;  $p < 0.02$ ). However, when spontaneous LH cycles were excluded, all the eggs were preovulatory for both the IVF 500 and 700 groups. Fertilization and cleavage were seen in 39 of 61 (63.9%) preovulatory oocytes in the IVF 700 group, and 42 of 91 (46.2%) in the IVF 500 group ( $p < 0.05$ ). Again when spontaneous LH cycles were excluded, there was no difference in fertilization and cleavage between the 2 groups. There was thus no difference in preovulatory egg morphology and quality in earlier administration of hCG in this clomiphene-hMG regime.

## INTRODUCTION

Follicular stimulation is one of the more critical aspects in any IVF program because it determines the egg quality. There are many regimes for follicular stimulation as discussed in earlier chapters, the most common being clomiphene-hMG (Seppala, 1985). In most stimulation regimes, hCG is usually administered for the final maturation (Seppala, 1985). However, some IVF centres still monitor for spontaneous LH surge, in the belief that this results in better quality eggs (Edwards, 1985). The incidence of LH surge is dependent on the stimulation regime and the duration before hCG is administered (see Chapter 6). Too early administra-



tion of hCG may result in premature luteinisation (see Brown, 1986); moreover, inadequate luteinization occurs due to lack of sufficient LH receptors (Channing and Kammermann, 1973). If hCG is administered too late, spontaneous LH surge may result and this is logistically unsatisfactory for many centres. We therefore investigated whether hCG given earlier resulted in better egg quality for IVF.

Another aspect in hCG administration is the length of the coasting period (ie the duration between the last hMG injection and the hCG injection). This was studied by Luafer, deCherney, Tartatzis et al (1984). They reported that delaying the hCG injection after hMG stimulation from 24 hours to 48-72 hours resulted in poorer fertilization (57% versus 84%) and an increase in degenerated oocytes (9% versus 1%).

#### PATIENTS AND METHOD

Patients in this study were from a subset of phase II, the stimulation and monitoring of which were discussed in Chapter 3. Hence only an emphasis on the characteristics of this sample is given here. Between November 1983 and June 1984, 27 patients (table 7.1) were stimulated with clomiphene citrate at 50 mg

twice a day from day 2. hMG was then administered in the afternoon at 2 ampoules per day from day 6 to 8. Subsequent doses were dependent on the plasma  $E_2$ , taken daily at 0830 hours. The patient was admitted when the  $E_2$  level reached 400 pg/ml/follicle. In the absence of spontaneous LH surge, hCG 10,000 iu was given early the morning after admission if the plasma  $E_2$  exceeded 700 pg/ml/follicle; a "coasting" period of at least 32 hours was allowed.

The second group of 30 patients (table 7.1) were recruited between July 1984 and December 1984. The stimulation and monitoring regimes were similar. HCG was given at 500 pg/ml/follicle in the absence of the LH surge.

Insemination of the eggs and culture of the embryos have been described in Chapter 3.

The data were analysed statistically using the Student's t-test.

## RESULTS

The mean number of hMG ampoules needed were 13.7 and 17.6 for the IVF 500 and IVF 700 groups respectively ( $p < 0.05$ ). The mean day

for laparoscopy was 13.2 and 13.3 respectively (n.s.;  $p > 0.05$ ). Plasma  $E_2$  levels reached 631 pg/ml/follicle and 682 pg/ml/follicle for the IVF 500 and IVF 700 groups respectively (ns;  $p > 0.05$ , table 7.2).

Spontaneous LH surge occurred in 8 and 14 cycles in the IVF 500 and IVF 700 groups respectively ( $p < 0.05$ ). As expected, the  $E_2$  values were higher on the day hCG was administered in the IVF 700 group ( $955 \pm 168$ , as opposed to  $690 \pm 196$  pg/ml/follicle for IVF 500;  $p < 0.001$ ). There was no such difference when spontaneous LH surge occurred.

There were more preovulatory eggs (mature and intermediate types) in the IVF 500 group ( $p < 0.02$ , table 7.3), reflected mainly in an increase in mature eggs (59.8% as opposed to 53.0% in the IVF 700 group). However, when eggs recovered after spontaneous LH surge were excluded, there were less mature eggs when hCG was given earlier (64.7% as opposed to 79.2% in the IVF 700 group). But there were no immature eggs obtained when spontaneous LH cycles were excluded.

In the IVF 500 group, the fertilization rate was less (46.2% of preovulatory eggs compared with 63.9% in the IVF 700 group, table 7.4;  $p < 0.05$ ). When there was no spontaneous LH surge, fer-



tilization and cleavage rates were similar regardless of timing of hCG administration.

There were 3 pregnancies in each group (table 7.4) but the numbers were too small for statistical evaluation. It was interesting to note that the pregnancies in the IVF 500 group were after hCG administration, while 2 of the 3 pregnancies in the IVF 700 group were after spontaneous LH surge.

## DISCUSSION

From the data it is clear that the incidence of spontaneous LH surge is dependent on when hCG is administered; the longer the delay, the more the likelihood of spontaneous LH surge. This may explain why spontaneous LH surges vary from 3-85%, with the median at 20% (Seppala, 1985). Trounson and Wood (1984) reported an LH surge incidence of 62.0% in clomiphene-hMG stimulated cycles.

Spontaneous LH surge resulted in eggs with better fertilization rates (56.0% as opposed to 42.6% when hCG was given in the IVF 500 group, and 66.7% as opposed to 45.8% in the IVF 700 group; ns, table 7.44). However, there were no pregnancies in 8 re-

placements with spontaneous LH surge in the IVF 500 group, while there were 2 pregnancies in 13 replacements in the IVF 700 group when spontaneous LH surge occurred. Trounson and Wood (1984) reported that the pregnancy rate per transfer with spontaneous LH surge was 23.1% as opposed to 12.3% when hCG was administered. The World Collaborative report showed that pregnancy rate was 15.6% with LH surge and 9.8% with hCG in clomiphene-hMG regimes (Seppala, 1985).

Steroid contents of follicular fluid show definite patterns of change evident towards ovulation, including a decrease in  $E_2$  and an increase in P (see Edwards, 1980). Such changes are also seen in follicular fluid following hCG administration; Templeton (1985) reported a decrease of  $E_2$  and androstenedione to 30% of pre-hCG levels, with a four-fold increase in P. Hence the micro-environment of the follicle is probably steroidogenically similar following an endogenous LH surge or hCG administration. This has significance in the morphological grading of eggs collected.

Morphological appearance of the cumulus and corona has been used to assess the maturity of the egg. Unfortunately, it is not representative of the egg maturation status (Laufer, Tarlatzis, de Cherney et al, 1984; Sundstrom and Nilsson, 1986). However, it is



still useful for assessing egg quality in any particular regime. Our data suggested that spontaneous LH surge resulted in more intermediate-quality eggs (12 of 25 [48.0%] in the IVF 500 group, and 21 of 42 [50.0%] in the IVF 700 group, ns; table 7.3). Also, in the absence of spontaneous LH surge, hCG administration resulted in more mature eggs morphologically, with more mature eggs obtained when there is a longer delay in hCG administration (44 of 68 [64.7%] in the IVF 500 group, and 19 of 24 [79.2%] in the IVF 700,  $p > 0.05$ ). This was not surprising since it has been reported that longer hCG-collection interval resulted in better egg recoveries, probably due to cumulus expansion (Templeton, 1985). However, in the mouse we have demonstrated that a shorter interval in collection after hCG did not result in poorer quality eggs (Edirisinghe, Law, Ng et al, 1986). In the absence of spontaneous LH surge, there was no statistical difference in fertilization and cleavage between the 2 groups (11 of 24 and 29 of 68 for IVF 700 and 500 respectively, table 7.4).

In natural cycles, the LH surge is dependent on  $E_2$  (Young and Jaffe, 1976; Marut, Williams, Cowan et al, 1981), produced mainly by the dominant follicle. In stimulated cycles, additional recruitment of developing ovarian follicles may result in an uneven cohort (see Hodgen, Kenigsburg, Collins et al, 1985). Our data was suggestive of closer cohort with hCG administration



in both the IVF 500 and IVF 700 groups. Hence it is possible that when spontaneous LH surge occurs, there may be a widening in egg maturity between the dominant follicle and the rest. However, it must be noted that while the maturity of the egg may be more advanced with hCG administration, fertilization and cleavage rates were better with spontaneous LH surge. Testart, Frydman, de Mouzon et al (1983) reported that the egg population was not homogenous when hCG was given.

In conclusion, spontaneous LH surge was more likely to occur when hCG administration was delayed. HCG administration resulted in more mature eggs assessed morphologically, but the incidence of fertilization and cleavage was less than in eggs recovered after spontaneous LH surge. In this clomiphene-hMG regime, earlier administration did not result in a change in the number of preovulatory eggs or fertilization and cleavage rate.

TABLE 7.1: AETIOLOGY OF INFERTILITY AND AGE OF PATIENT VERSUS hCG ADMINISTRATION

PATIENT -----	hCG ADMINISTRATION -----	
	IVF 500 (%) -----	IVF 700 (%) -----
1. Aetiology:		
Tubal	17 (56.6)	17 (63.0)
Tubal & oligospermic	6 (20.0)	3 (11.1)
Oligospermic	3 (10.0)	4 (14.8)
Idiopathic	2 ( 6.7)	1 ( 3.7)
Others	2 ( 6.7)	2 ( 7.4)
	-----	-----
	30	27
2. Age (years):		
35 and less	17 (56.7)	20 (74.1)
>35	13 (43.3)	7 (25.9)

TABLE 7.2: MEAN PLASMA ESTRADIOL LEVELS (pg/ml) PER FOLLICLE FOR PATIENTS WITH HUMAN CHORIONIC GONADOTROPIN AT DIFFERENT LEVELS

		Day prior to egg recovery @							
hCG		-----							
GROUP		7	6	5	4	3	2	1	0
-----		---	---	---	---	---	---	---	---
IVF 500 - M		82	124	171	224	346	527	631	399
	SD	20	32	53	64	105	154	197	128
IVF 700 - M		104	124	173	261	364	542	682	529
	SD	28	38	74	113	143	205	255	224

@ Estradiol values not statistically significant between IVF 500 and IVF 700 (p>0.05)



TABLE 7.3: EGGS COLLECTED FROM PATIENTS WITH HUMAN CHORIONIC GONADOTROPIN AT DIFFERENT LEVELS

EGG TYPE *	IVF 500			IVF 700		
	LH	hCG	subtotal	LH	hCG	subtotal
Mature	11	44	55	16	19	35
Intermediate	12	24	36	21	5	26
Immature	1	0	1	5	0	5
	25	68	92	42	24	66
n	8	19	27	14	8	22

\*  $p<0.02$  between oocyte types of IVF 500 and IVF 700

TABLE 7.4: EGGS FERTILIZED FROM PATIENTS WITH HUMAN CHORIONIC GONADOTROPIN AT DIFFERENT LEVELS

	IVF 500			IVF 700		
	LH	hCG	subtotal	LH	hCG	subtotal
Eggs coll *	25 (8) <sup>+</sup>	68 (19)	92 (27)	42 (14)	24 (8)	66 (22)
Fertilization	14 (8)	29 (13)	43 (21)	28 (13)	11 (8)	39 (21)
Pregnancies	0	3		2	1	

\* p<0.05 between pre-ovulatory eggs and fertilization of IVF 500 and IVF 700

+ number of patients in parenthesis

## CHAPTER 8:

### SPONTANEOUS LUTEINIZING HORMONE (LH) SURGES

#### SYNOPSIS

The presence of spontaneous LH surge is thought to be related to better pregnancy rates in clomiphene-hMG induced IVF cycles. LH in the urine has been easily detected with Hi-Gonavis. A retrospective analysis of 54 IVF cycles in which urine LH assayed by a microtitre method using Hi-Gonavis was compared to plasma LH assayed by RIA. The false negative rate was 7.4%, while the false positive rate was 16.7%. In the false negative group, hCG was given, after the LH surge in the plasma in all cases. Only 2 eggs were collected from 1 patient and both fertilized; none were collected from the other 3 patients because of spontaneous follicular rupture prior to laparoscopy. In the false positive group, 23 eggs were recovered from 9 patients. Fifteen (65%) of them were mature, and these fertilized and cleaved. On the other hand, in the true positive group (13 patients, 33 eggs), only 12 eggs (36%) were mature, and 19 (58%) fertilized and cleaved. The poorer results may be due to the absence of hCG injection.

#### INTRODUCTION



The release of endogenous gonadotropins is dependent on the pulsatile action of the hypothalamic GnRH on the pituitary. Hence its release is also cyclical (see chapter 2). However, when the negative feedback of  $E_2$  on the hypothalamus is converted to a positive feedback, there is a massive release of LH seen as a surge. Such surges differ in pattern in different species. In rats, there is a slow initial rise of LH followed by a linear increase to a peak persisting for about 2 hours (Blake, 1976). In mares, LH rises gradually throughout the cycle without a distinct surge, and ovulation occurs while the LH levels are still increasing (Nett, Pickett, Seidel et al, 1976). In women, the mid-cycle surge typically begins with a modest increase in plasma FSH and LH followed by a dramatic rise and an equally rapid fall. The LH peak is actually 2 peaks separated by an interval of 12-24 hours, with the whole discharge over in 30 hours (Edwards, 1980).

It is thought that the LH surge overcomes the locally inhibiting non-steroidal follicular factors preventing premature oocyte maturation, such as the oocyte maturation inhibitor, OMI (Tsafriri, Bar-Ami and Lindner, 1983). Approximately 20 hours after the LH surge, there is in vivo resumption of oocyte maturation; re-activation of meiosis from prophase of meiosis I results in extrusion of the first polar body between 25 and 35

hours after the LH surge (Edwards, 1980; Steptoe and Edwards, 1970; Testart, Thebault and Frydman, 1982b). The egg is then at metaphase II and is ready for fertilization by the sperm. These events can also be initiated by hCG, but its timing is important (see chapter 7). The significance of a spontaneous LH surge in an IVF program has also been discussed (see chapter 6).

While the LH surge has classically been detected with blood studies, it can also be detected in the urine, where it occurs soon after the surge in plasma. Urinary samples are preferred by the patients, and are usually collected over 24 hours. Immunoassays and receptor assays are widely used. Another fast assay method used clinically is an erythrocyte sedimentation assay (Hi-Gonavis, Mochida, Tokyo), which can be completed in 2-3 hours (Mizuno, Kawamoto, Mizukuchi et al, 1973; Yoshimoto, Moridera and Imura, 1974). Edwards, Anderson, Pickering et al (1982) have modified it into a microtitre method which has been claimed to be equally effective. The purpose of this chapter is to analyse data on the use of Hi-Gonavis for the detection of the spontaneous LH surge.

## MATERIALS AND METHOD

An analysis of 54 patients in phase II where there was adequate



plasma to determine the LH levels by radio-immuno-assay was made. The radio-immuno-assay method for LH has been previously described (Goh, 1981), and the incubation period takes 24 hours; the samples were collected together and analysed retrospectively. Only the Hi-Gonavis results were available for clinical decisions in the management of the patient. The stimulation and the monitoring have been dealt with in chapter 3.

The Hi-Gonavis assay is a kit assay which involves a step-wise dilution of urine. It is based on haemagglutination. If there is sufficient hCG or LH in the urine, the sheep erythrocytes agglutinate, and this modifies their sedimentation pattern in the ampoules. A positive reaction is seen as a smooth mass of cells at the bottom of the ampoule in 2 hours. The assay is sensitive to 12.5 iu LH or 5.0 iu hCG. A microtitre method was later devised, resulting in a four-fold savings of cost (Edwards, Anderson, Pickering et al, 1982). Tissue-culture grade microtitre plates were used. The urine was diluted with phosphate-buffered solution at a concentration of 65 ul of urine with 40 ul of PBS. Then 40 ul of the mixture was transferred into the next well and mixed with 25 ul of PBS, and this procedure was continued down the wells. The sensitized erythrocytes (100 ul) were then added into the wells after reconstituting in PBS containing bovine serum albumin. The wells were then shaken and left to stand for 2



hours. It was claimed that this method was more reliable and gave narrower serial dilutions, with greater precision.

Statistical analysis was carried out using the chi-square test.

## RESULTS

Of the 54 cases, there were 13 true positives (24.1%) and 28 true negatives (51.8%) (table 8.1). Four cases were false negatives (7.4%), while 9 were false positives (16.7%)

In the false negative group, hCG was given after the plasma LH surge because there was absence of the LH surge as detected by Hi-Gonavis. Of the 4 patients, 3 had spontaneous ovulation confirmed at laparoscopy and no eggs were recovered from them. In the 1 patient where there was no spontaneous ovulation, 2 eggs were recovered; one was mature and the other intermediate in quality. Both fertilized after insemination.

Of the 9 false positives, where there was no hCG given, the majority were pre-ovulatory (15 mature, and 5 intermediate, of 23 eggs). Only the 15 mature eggs fertilized (table 8.2).

In the true positive group (13 patients), where there was no hCG given, 12 of 33 eggs (36.4%) were mature, 16 intermediate and 5 poor (table 8.2). However only 19 fertilized (57.6%).

In the true negative group, there were 72 eggs from 28 patients. The majority (53 of 72) were mature, and 15 were intermediate eggs. However, the fertilization rate was the lowest (48.6%).

There were 4 pregnancies in this sample population, in 36 replacements (8.3%). Two were from the true positive, and 2 from the true negative groups.

## DISCUSSION

In the true positive group, all patients (with one exception) had the plasma LH rise coincidental with the detection of LH in the urine. One patient had an increase in plasma LH one day earlier than the Hi-Gonavis rise. At laparoscopy 3 follicles were already ruptured, and 1 unruptured follicle yielded 1 egg which fertilised and divided to 4 cells.

The Hi-Gonavis assay gave a high false positive of 16.7%. However, 65% of the eggs recovered from this group were mor-

phologically mature and were able to fertilize. Not surprisingly, there were no pregnancies in this group. Using Hi-Gonavis to detect LH surges in spontaneous cycles, Edwards and Steptoe (1974) reported failure in egg recovery of 35%, while Lopata, Johnston, Hoult et al (1980) reported a failure rate of 44%.

In spite of hCG injection, the true negative group had the lowest fertilization rate of 48.6%. It is possible that the hCG administration could have been too early in the majority of these cases. This was in contrast to other studies where hCG administration resulted in significantly improved embryo transfer rates (Umapathysivam, Jones and Meffin, 1986).

Because of the high false positive and false negative rates in the Hi-Gonavis assay, it was decided not to rely on it. In fact, because of the use of a combination of FSH-hMG stimulation, spontaneous LH surge are not detected now, mainly because this regime is supposed to have a low incidence of such surges.



TABLE 8.1: FALSE POSITIVES AND NEGATIVES WITH HI-GONAVIS

Plasma LH	Hi-Gonavis	
-----	-----	-----
	+	-
+	13	4 (FN)
-	9 (FP)	28
-----	-----	-----

FN = False negatives, 4/54 (7.4%)

FP = False positives, 9/54 (16.7%)

TABLE 8.2: QUALITY OF EGGS AND EMBRYOS OBTAINED CATEGORIZED ACCORDING TO SPONTANEOUS L.H. SURGE \*

HiG/Pl	Eggs(n)	Mature	Inter	Immat	Poor	Fert	Unfert
-----	-----	-----	-----	-----	-----	-----	-----
+/+	33	12(36%)	16(49%)	4(12%)	1(3%)	19(58%)	14(42%)
-/-	72	53(74%)	15(21%)	-	4(5%)	35(49%)	37(51%)
-/+	23	15(65%)	5(22%)	3(13%)	-	15(65%)	8(35%)
+/-	2	1(50%)	1(50%)	-	-	2(100%)	-

\* Statistically no difference between different egg gradings.

## CHAPTER 9:

### COMBINATION OF ULTRASOUND WITH ESTRADIOL

#### SYNOPSIS

There are various regimes of ultrasound monitoring of follicular growth for IVF. The majority are dependent on sequential monitoring. In this chapter, it was examined whether frequent ultrasonic monitoring of follicle growth is necessary for IVF. In phase I, 22 patients were stimulated with clomiphene citrate or were unstimulated, and had daily ultrasound scanning from day 8. In phase II, 28 patients were stimulated with clomiphene and hMG, and ultrasound scanning was reduced to once on day 9. Daily ultrasound in phase I showed a significant correlation in follicular growth in clomiphene-induced patients with 1 and 2 follicles ( $p < 0.001$ ), but there was no such correlation in unstimulated patients. In spite of reducing the scanning frequency to once per cycle in phase II, the numbers and side of follicles were correctly predicted in 60% (6 of 10 laparoscopies), as opposed to 30% (6 of 20 laparoscopies) in phase I. Spontaneous LH surge occurred in 13 of 25 phase II patients; the  $E_2$  values on



the day of the LH surge were higher when there were more follicles ( $r=0.69$ ). There were 7 preovulatory eggs of 11 eggs (63.6%) aspirated from phase I patients with tubal obstruction, and 26 of 30 eggs (86.7%) in phase II; cleavage rates of these embryos after fertilization were similar (85.7% and 73.1% respectively). Although pregnancy rates in patients with tubal obstruction were better in phase II (3 of 9 replacements) than in phase I (1 of 7 replacements), this was probably a reflection of multiple embryo replacement in phase II. It is concluded that it is not necessary to do frequent ultrasound scanning to monitor follicular growth for IVF patients stimulated with clomiphene-hMG.

## INTRODUCTION

Monitoring of follicular growth is important in IVF programmes. Most centres use a combination of ultrasound and  $E_2$  assays (Trounson and Conti, 1982) though some use either alone. Ultrasound monitoring as the only method of monitoring follicular growth has been reported by a few centres (Hoult, de Crespigny, O'Herlichy et al, 1981; Little, Bromwich, Walker et al, 1983; Smith, Porter, Craft et al, 1984). Bourn Hall clinic is one of the very few centres using endocrine parameters

(urinary estrogens) without ultrasound (Edwards, Steptoe and Purdy, 1980), although recently they are relying on ultrasound scans as well.

There are various regimes of ultrasound monitoring of follicular growth. Most centres begin monitoring by day 10 of the cycle, though some advocate adequate response (eg. by  $E_2$ ) before monitoring (Kerin, Edmonds, Warnes et al, 1981). Scanning can be done daily or intermittently. Sequential scanning gives a better picture of follicular growth and change in its growth rate or achieving a minimum diameter or volume may be used as an index of maturity. Some IVF groups give hCG for final follicular maturation on this basis (Hoult, de Crespigny, O'Herlichy et al, 1981). Intermittent scanning is adequate for IVF groups not relying on ultrasonic parameters of follicular maturation (Buttery, Trounson, McMaster et al, 1983). Its use then may be confined to determination of the number or the site of follicles. Under such circumstances, follicular growth is monitored usually by  $E_2$ .

The IVF program at KK Hospital had 2 main stimulation regimes. The use of ultrasound scans in follicular scanning in these 2 regimes were therefore investigated. For the first nine months (phase I), patients were stimulated with clomiphene citrate or

were unstimulated, and were monitored with daily ultrasound scanning and daily plasma  $E_2$  from day 9. From September 1983 (phase II), patients were stimulated with clomiphene and hMG, and were monitored with daily plasma  $E_2$  from day 8; ultrasound scanning was reduced to once on day 9, and a repeat later if necessary.

#### PATIENTS AND METHOD

In phase I, there were 22 patients involved in 33 patient cycles, with 22 laparoscopic recoveries. In phase II the first 28 patients (comparable sample size) in 28 cycles (with 25 laparoscopic recoveries) were studied. The method of stimulation and monitoring have been described in chapter 3.

#### Ultrasound scanning

This was done mainly by one ultrasonographer throughout the period of study. In phase I it was done daily from day 8, while in phase II scanning was done once in day 9 and repeated (usually on day 10 or 11) if necessary. Follicles above 5 mm were counted. The ultra-sound machine was a Hitachi linear array model EUB 25 with a 3.5 mHz probe.



### Statistical analysis

There was a great variation in the average follicle diameters. Hence, in the statistical analysis of follicular growth, the median values instead of the mean values were used. The correlation was calculated using non-parametric methods (Spearman's correlation). The regression equation was calculated for the  $E_2$  levels at which spontaneous LH occurred.

### RESULTS

In phase I, follicular measurements were available daily from day 8. On each day, up to laparoscopy, there was a wide variation in the average diameter of follicle between patients. The median values are shown in table 9.1. In clomiphene-stimulated cycles, there was a significant correlation in growth pattern in patients with 1 and 2 follicles ( $p < 0.001$ ). For the 3 and 5 follicle groups, the numbers were too small for meaningful interpretation. In the unstimulated cycles, there was no such correlation in patients with 2 follicles, signifying perhaps an unsatisfactory growth pattern. Though there was a correlation for the unstimulated group with 1 follicle, the number of patients was too small

to draw any conclusion.

The numbers and side of follicles were correctly predicted by ultrasound in 30% (6 correct in 20 laparoscopies where the ovaries could be seen clearly) in phase I, and 60% (6 correct in 10 laparoscopies) in phase II. This difference was not significant (n.s.; Fisher's exact probability test). There were severe adhesions in the other 15 laparoscopies done in phase II. Preovulatory eggs collected from patients with tubal obstruction were 7 of 11 eggs (63.6%) in phase I, and 26 of 30 eggs (86.7%) in phase II ( $p < 0.05$ ). The quality of the eggs was similar in both phases, as evidenced by cleavage rates of 85.7% (6 of 7) in phase I and 73.1% (19 of 26) in phase II (ns,  $p < 0.05$ ). Eggs collected from patients with oligozoospermia and prolonged idiopathic subfertility were excluded because fertilization may be diminished in these 2 groups (Mahadevan, Trounson and Leeton, 1983). Pregnancy rates for patients with tubal obstruction were better in phase II (33.3%, 3 of 9 replacements) than in phase I (14.3%, 1 of 7 replacements).

Of the cases that had laparoscopy in phase I, 14 of 22 (63.6%) had spontaneous LH surge, while in phase II, 13 of 25 (52.0%) had spontaneous LH surge (n.s.). The  $E_2$  values on the day of spontaneous LH surges in phase II were higher when there were more

follicles (figure 9.1), with a regression equation

$$Y = 820.8 + 380.3X$$

where Y = plasma E<sub>2</sub> value (pg/ml) at which spontaneous LH surge would occur, and X = number of follicles on ultrasound scan on day 9. The correlation coefficient, r is 0.69, at 9 degrees of freedom (p<0.05).

## DISCUSSION

Follicular growth patterns could be studied only in phase I. Unstimulated cycles did not show significant correlation in growth patterns, and this may indicate unsatisfactory follicular growth. Clomiphene-stimulated cycles with 1 and 2 follicles showed a significant correlation in growth pattern. There were inadequate sample sizes in clomiphene-stimulated cycles with more than 2 follicles. The median diameters were much smaller in the clomiphene-induced cycle in which 5 follicles developed (table 9.1).

In spite of good growth correlation patterns for phase I, the accuracy of ultrasound scan of follicles (as confirmed by



laparoscopy) was only 30%. In phase II when the ultrasound scan was limited to day 9, the accuracy was 60%. This improvement may be attributed to experience though there was significant growth coefficient in phase I for 1 and 2 follicles. Operator variability may have also been contributory (Prins and Vogelzang, 1984).

Spontaneous LH surge occurred in 52.0% of phase II patients. Based on the regression equation (figure 9.1), it is possible to give hCG just before the anticipated spontaneous LH surge, thereby being closer to the natural state and yet minimizing the inconvenience of spontaneous LH surge. In unstimulated cycles, blood  $E_2$  levels can be used to predict the LH surge (McIntosh, Mathews, Crocker et al, 1980). However, in the presence of multiple follicles of different cohorts, daily ultrasound scans should be able to anticipate the likelihood of spontaneous LH surge better than  $E_2$  values. Buttery, Trounson, McMaster et al (1983) have calculated a regression equation to determine the LH surge based on the diameter of the largest follicle. Levran, Lopata, Nayudu et al (1985) reported in a study of 117 women stimulated with clomiphene-hMG that in those cycles where a spontaneous LH surge occurred, most surges were detected on the seventh day of  $E_2$  rise.

The quality of eggs recovered was better in phase II when the ultrasound scan frequency was reduced to once-a-cycle; 86.7% was pre-ovulatory as opposed to 63.6% in phase I. Laufer, de Cherney, Haseltine et al (1983) reported recovery of 42% mature and 49% intermediate eggs (based on the morphological appearance of the oocyte-corona-cumulus complex) in hMG-stimulated cycles with daily ultrasound scans. Cleavage rates in our series were 85.7% and 73.1% of pre-ovulatory eggs in phase I and II respectively. This was comparable to 80.1% cleavage rate in tubal obstruction reported by Mahadevan, Trounson, Leeton et al (1983), and to 68% for mature eggs and 79% for intermediate eggs in the series of tubal disease of Laufer, de Cherney, Haseltine and co-workers (1983). The improved pregnancy rate in phase II was probably a reflection of multiple embryo replacement (1.1 embryos per replacement in phase I and 2.3 embryos per replacement in phase II).

Frequent ultrasonic scans for follicles interfere with urine collection (necessary for detection of spontaneous LH surge); timing became variable with excessive urine volumes. The latter was a disadvantage when Hi-Gonavis was used to monitor for spontaneous LH surge. However, these surges occur more commonly in clomiphene-hMG cycles and rarely in hMG-alone cycles for IVF (see Chapter 6). Thus urine monitoring for LH is not necessary for



hMG-alone cycles. It is possible to circumvent the high urine output by measuring blood LH, but this requires more venepunctures or an indwelling cannula.

Another possible disadvantage of repeated ultrasonic scan is ultrasound-induced damage to eggs during meiotic reactivation (Edwards, 1984). However, ultrasonically-guided egg recovery has been practised since 1981 (Lenz, Lauritsen and Kjellow, 1981). Recently there has been some reports of pregnancies with this technique (Wikland, Nilsson, Hansson et al, 1983; see Seppala, 1995) and many normal deliveries have occurred without being reported yet in the literature. In addition to pregnancies from transvesical aspiration, the transvaginal route has also been successful (Dellenbach, Nisand, Moreau et al, 1984). Caution is also advised with regards to ultrasound scans for follicular maturity because premature rupture of mature follicles has been reported (Testart, Thebault and Frydman, 1982a).

In this study, reducing the ultrasound scan frequency to once-a-cycle did not compromise egg quality. In fact, it improved the accuracy rate under those circumstances. Following that study minimal ultrasound scanning for the rest of phase II, usually once a cycle, was carried out. It must be noted that at that time two leading IVF centres did not scan their patients



frequently: Bourn Hall clinic (Edwards, Steptoe and Purdy, 1980) and Monash University (Buttery, Trounson, McMaster et al, 1983).

However, when we started on the FSH-hMG (Metrodin-Pergonal) combination we found that  $E_2$  alone was inadequate to monitor the progress of such cycles, mainly because the number of follicles were much more in excess of patients stimulated with clomiphene-hMG. For this current stimulation regime, ultrasound scans are done once every other day from day 7 until the follicles reach about 14 mm in diameter, when the scans are repeated daily until egg recovery.

Ultrasound assessment of follicular growth is useful in identifying the ovary in which the follicle(s) may be developing. This is important for centres where there are no facilities for ultrasonic-guided aspiration of follicles. Under such circumstances, it is useful to identify patients in whom one ovary may be covered by adhesions. It also helps clarify the response to stimulation and may assist in deciding the optimal time to start hormonal assays. Kerin, Edmonds, Warnes et al (1981) concluded that a diameter of 12 mm for the largest follicle was an indication to start hormonal monitoring.

Ultrasound cannot be used as the sole monitor to determine the

time of injection of hCG because by itself ultrasound does not adequately assess the functional capacity of the follicles (Trounson, 1982). Also, though the presence of a cumulus on ultrasound has been reported to be reliable (Hackeloer and Hansmann, 1983), other workers have found its presence unreliable (Nitschke-Dabelstein, 1983). However, there have been some reports on the reliance of ultrasound scans to determine follicle maturity and timing of hCG, based mainly on the echogenicity of the endometrium (Smith, Porter, Craft et al, 1984)

TABLE 9.1: MEDIAN VALUES OF AVERAGE FOLLICLE DIAMETERS (cm) IN CLOMIPHENE-INDUCED AND UNSTIMULATED CYCLES

Follicle numbers	Days before laparoscopy								n	r
-----	-7	-6	-5	-4	-3	-2	-1	0	--	-----
-----	---	---	---	---	---	---	---	---		

Clomiphene

One	1.20	1.40	1.60	1.93	2.17	2.23	2.33	2.27	7	0.976
Two	1.13	1.20	1.37	1.47	1.72	1.90	2.10	2.02	12	0.976
Three	-	-	-	-	1.60	1.68	2.00	1.80	6	0.800
Five	-	0.90	-	1.20	1.20	1.53	1.37	1.25	5	0.753

Unstimulated

One	-	0.97	-	-	1.14	1.29	1.52	1.93	2	1.000
Two	-	1.44	2.23	1.23	1.39	1.50	1.78	1.57	10	0.250

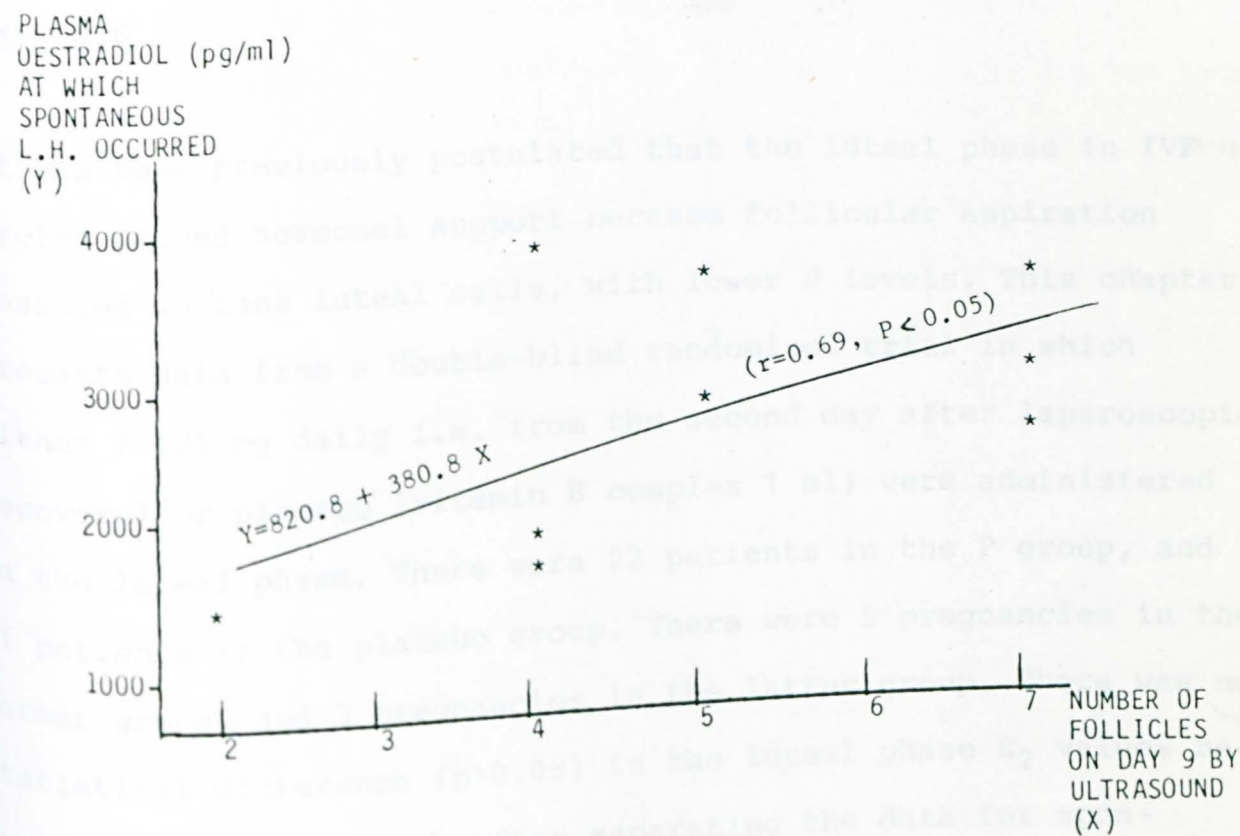
-----

n = number of follicles

r = Spearman's correlation coefficient



FIGURE 9.1: ESTRADIOL VALUES IN CLOMIPHENE-HMG INDUCED PATIENTS WHEN SPONTANEOUS LUTEINIZING HORMONE SURGE OCCURRED.



## CHAPTER 10

### LUTEAL PHASE MANAGEMENT

#### SYNOPSIS

It has been previously postulated that the luteal phase in IVF cycles needed hormonal support because follicular aspiration resulted in less luteal cells, with lower P levels. This chapter presents data from a double-blind randomized trial in which either P (25 mg daily i.m. from the second day after laparoscopic recovery) or placebo (vitamin B complex 1 ml) were administered in the luteal phase. There were 22 patients in the P group, and 21 patients in the placebo group. There were 5 pregnancies in the former group, and 3 pregnancies in the latter group. There was no statistical difference ( $p>0.05$ ) in the luteal phase  $E_2$  values between the 2 groups, even after separating the data for spontaneous LH surge and hCG administration. However, there was a significant difference ( $p<0.02$ ) in the plasma P on days 10 and 12, when the patients in the placebo group had lower plasma P, and this was contributed mainly by patients who had spontaneous

LH. Luteal phase duration was significantly shorter in the placebo group as menses was prevented from coming in the P group (13.0 days and 14.8 days respectively,  $p < 0.001$ ). Higher  $E_2$  levels on the day before laparoscopic egg recovery did not shorten the luteal phase.

## INTRODUCTION

The corpus luteum is intimately dependent on pre-ovulatory follicular development. After extrusion of the egg, capillaries from the thecal layers invade the granulosa cell layer and fill the former antral cavity with blood. Cholesterol transported by low density lipoproteins is thus allowed to reach the granulosa cells to augment progesterone production (Carr, MacDonald and Simpson, 1982). Early luteal function is dependent on basal levels of LH. This is classically seen in ovulation induction in hypogonadotropic hypogonadic patients, who exhibit shortened luteal phases when a single LH dose was administered for the pre-ovulatory gonadotropin surge, but when hCG or repeated LH doses were given there was basal gonadotropic support of the corpus luteum (Jewelwicz, Dyrenfurth, Warren et al, 1973).

P secretion affects both the ovarian and endometrial development.



Together with  $E_2$ , P exerts a negative feedback on gonadotropin secretion. New follicular development is further suppressed by antagonism of  $E_2$  dependent mitogenic effects of other follicles. McNatty, Hillier, van der Boogaard, et al (1983) reported that 94% of luteal phase follicles greater than 1 mm in diameter were atretic (as determined by oocyte viability and granulosa cell numbers). Goodman and Hodgen (1977, 1979) noted that ovulation occurred in monkeys in the contralateral ovary in about 70% of the time in normal menstrual cycles. Also, regardless of the site of the former corpus luteum, the subsequent dominant follicle develops in the ovary which produced the lowest amount of P into the venous system during the antecedent luteal phase (diZerega and Hodgen, 1982). Hence, intraluteal P affects the future development of the follicle. However Baird, Backstrom, McNeilly et al (1984) reported that the absence of healthy antral follicles in the luteal phase was due to inhibitory effects of the corpus luteum. They postulated that the suppression of gonadotropins is by ovarian steroids secreted by the corpus luteum.

Approximately 8 days after ovulation, hCG from the developing trophoblast can rescue the corpus luteum. A 2-week prolongation of the corpus luteum's lifespan (and hence, the menstrual cycle) can be elicited experimentally with exogenous hCG injections ap-

proximately 8 -10 days after ovulation (Quagliarello, Goldsmith, Steinetz, et al, 1980). Since hCG alone cannot simulate pregnancy maintenance of the corpus luteum beyond this 2-week interval, other intrinsic factors may be present. Furthermore, it is known that the sensitivity of the corpus luteum to LH/hCG stimulation progressively decreases as a non-steroidal LH receptor binding inhibitor (LHRBI) increases (Channing, Anderson, Hoover, et al, 1982). P secretion in vitro can be inhibited by LHRBI. Maintenance of the corpus luteum in early pregnancy may result from a non-hCG mediated antagonism of the effect of LHRBI.

P support in the luteal phase is controversial. Work with primates suggest compromised P production in a third of them (Kreitmann, Nixon and Hodgen, 1981). However, clinical studies have not been convincing (Feichtinger, Kemeter, Szalay et al, 1982; Garcia, Jones, Acosta et al, 1981). Yovich has reported the use of oral medroxyprogesterone acetate (Yovich, Puzey, de'Atta et al, 1982) and hCG (Yovich, Stanger, Yovich et al 1984b). However, most centres do not give such therapy (Trounson and Wood, 1981). The purpose of this chapter was to study the differences between P support and placebo support in the luteal phase of IVF patients stimulated with clomiphene-hMG.



## MATERIALS AND METHODS

Patients were from phase II of the IVF program. The stimulation and monitoring have been described in chapter 3.

Following laparoscopic egg recovery, the patients were randomized in a double-blind study into 2 groups: one group was given i.m. P 25 mg daily from the 2nd post-recovery day until the 14th post-recovery day, and the other group was given i.m. vitamin B complex injection 1 ml (placebo group) for the same duration. The injections were covered and neither the patient nor the nurse giving the injection knew what was administered. The code was broken only after the outcome of the treatment was known. There was a total of 22 patients in the P group, and 21 patients in the placebo group.

Serum was taken once every 2 days in the morning before the injections starting from the day of laparoscopic egg recovery. The samples were collected and analysed together. After the 10th day following laparoscopic egg collection, the values from pregnant patients were not included in the analysis.

Statistical analysis was by the Student's t-test.



## RESULTS

The plasma  $E_2$  results are given in table 10.1. The  $E_2$  level for the group given P rose from a mean of 777.3 pg/ml on the day of egg collection to 1451.1 pg/ml on the 4th day after the recovery (day 4). It then declined to 105.4 pg/ml on day 14. The  $E_2$  values for placebo group were similar, but with a gentler decline on day 6 and day 8. However, there were no statistical difference between the 2 groups ( $p > 0.05$ ).

Plasma P values are given in table 10.2. As with  $E_2$ , P values increased from 14.1 and 21.7 ng/ml on the day of collection to a maximum on day 4 (56.4 and 53.5 ng/ml for the P and placebo group respectively). There was no statistical difference between the 2 groups in the first 8 days although the plasma P was slightly lower in the former group inspite of exogenous P (except day 4). However, on the 10th and 12th day, the plasma P was significantly higher in the P group ( $p < 0.02$ ); on the 14th day, it was also higher but this was not significant ( $p > 0.05$ ). There was an extremely high P value of 86.6 ng/ml on day 14 in the P group without pregnancy and this value was excluded in the analysis.

When spontaneous LH surge cases were analysed separately from the

hCG patients, there was no statistical differences in plasma  $E_2$  for the groups given P or placebo (tables 10.3 and 10.4). There was similiarly no difference in the plasma P (tables 10.5 and 10.6) except for the spontaneous LH group where plasma P was significantly lower in days 10, 12 and 14.

Plasma P values also did not differ significantly ( $p > 0.05$ ) when the hCG subgroup was compared with the spontaneous LH subgroup for both the groups given P or placebo.

Treatment with either P or placebo did however significantly affect the luteal phase duration (table 10.7). Menses was prevented until a mean of 14.8 days after egg recovery in the P group, after the P was stopped. In the placebo group, the luteal phase length of 13.0 days was shorter ( $p < 0.001$ ). To check whether higher pre-recovery  $E_2$  resulted in a shorter luteal phase, the data was analysed with  $E_2$  values on the day before laparoscopic egg recovery at 2000 pg/ml or less, and above 2000 pg/ml. There was no significant shortening ( $p > 0.05$ ) of the luteal period in both the P and the placebo groups (table 10.7).

There were 5 pregnancies in the P group (1 biochemical, 2 abortions, and 2 full-term pregnancies), and 3 pregnancies in the placebo group (1 abortion and 2 full-term pregnancies). The num-



ber of pregnancies were too small for meaningful statistical analysis, but there is no obvious advantage in P therapy in so far as pregnancies were concerned.

## DISCUSSION

In the series of 43 patients stimulated with clomiphene-hMG, there was an artificial prolongation of luteal phase duration in the supported group because of P administration until the 14th day after laparoscopic egg recovery. In the placebo group, the duration was only  $13.0 \pm 1.4$  days. This was much shorter than the mean of  $15.5 \pm 2.3$  days reported for clomiphene-hMG cycles without luteal phase support (Cohen, Debauche, Pigeau et al, 1984) and the mean of  $15.1 \pm 1.4$  days reported for hMG-stimulated IVF cycles without luteal phase supplementation by Garcia, Jones, Acosta et al (1981). However, the values were equivalent to the mean of  $13.5 \pm 1.3$  days reported for unstimulated IVF cycles by Feitchtinger, Kemeter, Szalay et al (1982) and  $12.3 \pm 3.5$  days for the non-aspirated controls reported by Garcia, Jones, Acosta et al. Clomiphene-induced cycles were reported to have normal luteal function (Trotnow, Becker, Kniewald et al, 1982; Kerin, Warnes, Quinn et al, 1983a).



In our series, the maximum mean P value for the placebo group was on the fourth day after the laparoscopy (53.5 ng/ml  $\pm$ 31.2). This corresponded to the report of Gronow, Martin, Hay et al (1985) where they monitored 55 patients through the luteal phase with plasma E<sub>2</sub> and pregnanediol glucoronide every other day; both conceptual (11 pregnancies) and non-conceptual cycles showed a peak in pregnanediol glucoronide level around luteal day 6; the level then fell and was "rescued" if there was an implanting embryo. In Garcia, Jones, Acosta et al's (1981) series, the maximum P value was around 19 ng/ml on the 7th day after laparoscopy for the hMG-stimulated IVF cycles without luteal phase supplementation. There was no luteal phase defects in this group. The gross difference in P values cannot be explained by the different stimulation regimes. In a report on 13 women stimulated with clomiphene citrate, Oskowitz, Seibel, Smith et al (1986) found that there was no difference in mean P levels in the group of women who had follicular aspiration, compared to the control group (no aspiration). They also found that in the first luteal week, the clomiphene group had higher levels of P, compared to an unstimulated group; in the second week P levels were similar. Our data also showed no demonstrable luteal phase deficiency in the placebo group. In their study of 32 non-stimulated cycles, Feichtinger, Kemeter, Szalay et al (1982) reported a deficiency of the luteal phase in 2 cases.

A mature Graafian follicle has about  $50 \times 10^6$  granulosa cells (McNatty, Smith, Makris et al, 1979). On follicular aspiration, an average of  $3 \times 10^6$  are removed (Jones, Garcia and Acosta, 1981); this is less than 10% of the total content. However, the authors reported that in 9 spontaneous cycles (with LH surge), multiple vigorous aspirations were done in a futile effort to obtain oocytes; the level of P in the luteal phase in these patients were significantly less than the controls (where no laparoscopy was done).

In the P group, the length of the luteal phase ( $14.8 \pm 0.7$  days) was much shorter than a similar group reported by Garcia, Jones, Acosta et al (1981) ( $19.6 \pm 3.9$  days). This may be due to continued P injections. Unfortunately, the authors did not specify the duration of the luteal phase support, and it is not possible to interpret the data on luteal phase length. However, their reported group of 8 patients with luteal phase support included one patient who was given hCG. Yovich, Stanger, Yovich et al (1984b) reported that hCG promotes a longer luteal phase duration than medroxyprogesterone acetate. In a controlled trial with intramuscular 17-OH progesterone caprioate (Proluton), Leeton, Trounson and Jessup (1985) reported that there was no increase in pregnancy rates with Proluton; there was no data on luteal phase



length or hormonal levels in that report. The maximum mean P value in P-supported group in the present study was 56.4 (+24.1) ng/ml on the 4th day following laparoscopy, whereas for Garcia, Jones, Acosta et al's (1981) series the maximum mean value was around 21 ng/ml on the 8th day.

Estrogen administered early in the luteal phase increases the ratio of prostaglandin F to prostaglandin E (Balmaceda, Asch, Fernandez et al, 1980), an event occurring physiologically late in the luteal phase (Balmaceda, Valenzuela, Eddy et al, 1980). It has also been reported that prostaglandin F<sub>2a</sub> inhibits P production while prostaglandin E<sub>2</sub> stimulates P synthesis (Sotrel, Helvacioğlu, Dowers, et al, 1981; Peters and McNatty, 1980). It is possible that luteal E<sub>2</sub> fosters the decline of the corpus luteum via alteration of this prostaglandin balance. This is seen clinically when the follicular E<sub>2</sub> levels rise excessively after stimulation (Olson, Rebar, Schreiber et al, 1983). However, in the small series of the present study, there was no shortening of luteal phase duration when the pre-recovery E<sub>2</sub> was high (above 2000 pg/ml). Since hCG inhibits prostaglandin synthesis (Balmaceda, Asch, Fernandez et al, 1981), the elevation of prostaglandin F<sub>2</sub>:prostaglandin E may be prevented in the corpus luteum by the intervening pregnancy.



In this study it was demonstrated that there was no difference in  $E_2$  levels in the luteal phase. This was also reported for hMG-stimulated patients (Muasher, Acosta, Garcia et al, 1984). They reported that the mean  $E_2$  and P during the luteal phase showed no statistically significant difference between the pregnant and non-pregnant patients except after day 11. The mean serum  $E_2$  in most of the luteal phase days was highest in the high responders followed by the normal and low responders.

There was a significantly higher plasma P in the P supported group on days 10, 12 and 14. This was seen only in the patients with spontaneous LH surges, and not after hCG injection. There was also no significant improvement in pregnancy rates with P support. Hence, there was no advantage in giving P. However, it has been reported by Jones (1983) that their stimulation with gonadotropins has to be complemented with P or hCG in the luteal phase because endogenous LH production is not sufficient for the high number of receptor sites.

Changes in luteal phase endometrium after stimulation with clomiphene-hMG has been reported by Cohen, Debauche, Pigeau et al (1984). In 19 cycles where no embryo replacement was done due to adhesions preventing puncture, infected or insufficient sperms, failure of fertilization, or failure of cleavage, 7 were within

normal limits, 8 were hypotrophic (with inability to maintain nidation), and 4 were intermediate. When the endometrial quality biopsied on the third day after laparoscopy was compared with estradiol and progesterone on the same day in 12 cases, highly variable steroid levels were observed in both competent and incompetent endometrium. Hence it is not possible to predict the state of the endometrium on steroid levels alone. In another study with clomiphene-stimulated cycles (Frydman, Testart, Giacomini et al, 1982), histologic parameters coincided with the luteal phase day, in contrast to spontaneous cycles where 24% of the cases who had endometrial biopsies had dystrophic endometrium. Abate, Call and Stanchi (1984) reported that endometrial biopsy done at the time of embryo replacement had no effect on pregnancy results; pregnancy rate was highest when the endometrial histology had a 17th or 18th day pattern. However, on ultrasonography in the periovulatory phase, there was a better chance of implantation if the endometrium was thick (Glissant, de Mouzon and Frydman, 1985). Interestingly too, they found no correlation between the thickness of the endometrium and follicle numbers; the same conclusion was also found for hormonal levels.

TABLE 10.1: PLASMA ESTRADIOL LEVELS (pg/ml) IN THE LUTEAL PERIOD  
WITH PROGESTERONE SUPPORT OR PLACEBO TREATMENT

		Days after egg recovery							
		0	2	4	6	8	10	12	14
		-----	-----	-----	-----	-----	-----	-----	-----
<u>Progesterone</u>									
Mean		777.3	1198.0	1451.1	1101.1	610.7	247.8	126.3	105.4
SD.		536.3	1056.3	1160.0	780.5	536.1	213.5	41.3	18.2
n		22	22	22	22	22	17	17	15
<u>Placebo</u>									
Mean		691.3	1119.6	1531.7	1438.8	890.0	278.4	145.1	109.0
SD.		427.9	678.2	1178.6	981.6	554.7	109.3	70.9	34.2
n		21	21	21	21	21	18	14	4

P values were not significant on all days (p>0.05)



TABLE 10.2: PLASMA PROGESTERONE LEVELS (ng/ml) IN THE LUTEAL PERIOD WITH PROGESTERONE SUPPORT OR PLACEBO TREATMENT

		Days after egg recovery							
		0	2	4	6	8	10	12	14
<u>Progesterone</u>									
Mean		14.1	38.4	56.4	43.9	25.6	15.5	11.1	12.0
SD.		0.0	17.0	24.1	22.3	25.1	10.2	5.6	4.9
n		22	22	22	22	22	17	17	15
<u>Placebo</u>									
Mean		21.7	43.0	53.5	52.1	28.4	8.6	5.2	7.6
SD.		38.3	25.6	30.3	23.7	17.3	4.0	5.1	8.2
n		21	21	21	21	21	18	14	4
p		ns	ns	ns	ns	ns	<0.02	<0.01	ns

TABLE 10.3 PLASMA ESTRADIOL LEVELS (pg/ml) IN THE LUTEAL PERIOD  
 FOLLOWING HCG INJECTIONS WITH PROGESTERONE SUPPORT OR  
 PLACEBO TREATMENT

	Days after egg recovery							
	0	2	4	6	8	10	12	14
<hr/>								
<hr/>								
<u>Progesterone</u>								
Mean	855.7	1438.5	1725.1	1303.9	707.1	258.4	136.7	98.5
SD.	564.6	1182.7	1314.7	829.8	600.9	249.3	42.6	33.3
n	15	15	15	15	15	12	12	10
 <u>Placebo</u>								
Mean	795.0	1439.5	2054.5	1879.4	1170.2	286.2	141.7	135.5
SD.	523.0	727.2	1279.5	1024.6	574.5	109.7	65.3	26.2
n	12	12	12	12	12	9	8	2
<hr/>								

p values were not significant on all days (p>0.05)

TABLE 10.4: PLASMA ESTRADIOL LEVELS (pg/ml) IN THE LUTEAL PERIOD  
 FOLLOWING SPONTANEOUS L.H. SURGE WITH PROGESTERONE SUPPORT  
 OR PLACEBO TREATMENT

	Days after egg recovery							
	0	2	4	6	8	10	12	14
<hr/>								
<u>Progesterone</u>								
Mean	666.4	682.8	719.9	666.4	404.1	222.4	101.4	101.0
SD.	395.8	432.7	378.7	449.8	300.5	101.4	26.9	22.2
n	7	7	7	7	7	5	5	5
 <u>Placebo</u>								
Mean	515.1	693.0	834.7	851.2	516.4	270.6	149.5	82.5
SD.	140.2	253.9	520.9	534.2	201.6	115.0	84.0	3.5
n	9	9	9	9	9	9	6	2
<hr/>								

p values were not significant on all days (p>0.05)



TABLE 10.5: PLASMA PROGESTERONE LEVELS (ng/ml) IN THE LUTEAL PERIOD FOLLOWING HCG INJECTIONS WITH PROGESTERONE SUPPORT OR PLACEBO TREATMENT

	Days after egg recovery							
	0	2	4	6	8	10	12	14
<hr/>								
<u>Progesterone</u>								
Mean	17.2	44.9	65.6	50.1	34.6	16.5	11.4	12.7
SD.	10.6	17.0	22.8	23.3	29.2	11.9	6.3	5.5
n	15	15	15	15	15	12	12	10
<hr/>								
<u>Placebo</u>								
Mean	28.7	54.6	66.9	62.1	36.5	9.2	6.1	11.0
SD.	48.3	27.0	33.4	24.9	17.3	4.3	6.7	12.2
n	12	12	12	12	12	9	8	2

P values were not significant on all days ( $p > 0.05$ )

TABLE 10.6: PLASMA PROGESTERONE LEVELS (ng/ml) IN THE LUTEAL PERIOD FOLLOWING SPONTANEOUS L.H. SURGE WITH PROGESTERONE SUPPORT OR PLACEBO TREATMENT

	Days after egg recovery							
	0	2	4	6	8	10	12	14
<u>Progesterone</u>								
Mean	7.4	25.4	36.7	30.8	18.9	12.9	10.2	10.3
SD.	3.4	6.7	12.4	13.5	4.9	3.7	4.1	0.1
n	7	7	7	7	7	5	5	4
<u>Placebo</u>								
Mean	12.2	27.5	37.1	38.8	17.5	8.1	4.0	4.3
SD.	10.0	12.8	14.7	14.4	10.2	3.8	1.3	3.0
n	9	9	9	9	9	9	6	2
p	ns	ns	ns	ns	ns	p<0.05	p<0.01	p<0.01

TABLE 10.7: LUTEAL PHASE DURATION

Treatment with progesterone*	:	14.8 $\pm$ 0.7 days
Treatment with placebo*	:	13.0 $\pm$ 1.4 days

Estradiol before egg recovery<sup>+</sup>

Treatment with progesterone :

< 2000 pg/ml :	14.5 $\pm$ 1.0 days
> 2000 pg/ml :	15.0 $\pm$ 0.0 days

Treatment with placebo :

< 2000 pg/ml :	13.2 $\pm$ 1.6 days
> 2000 pg/ml :	12.8 $\pm$ 1.2 days

\*  $p < 0.001$ ; +  $p = \text{n.s.}$



## CHAPTER 11:

### CONCLUSION

Based on the information analysed, the following overall conclusions can be drawn:

1. The mean follicular phase  $E_2$  levels per follicle in the unstimulated and stimulated patients (clomiphene and clomiphene-hMG) were similar.
2. There were more follicles seen on ultrasound with the clomiphene-hMG stimulation, though there was no difference in administering 1 or 2 ampoules/day from days 6 to 8.
3. Though there were more pre-ovulatory eggs collected when the hCG was given earlier, the fertilization and cleavage rates were reduced. However, this effect was probably due to the higher incidence of spontaneous LH surge when hCG was delayed because there was no statistical difference in the egg quality and fertilization when the patients with spontaneous LH surge were excluded.
4. Spontaneous LH surge measured with Hi-Gonavis had a high false

negative (7.4%) and a high false positive (16.7%) rate. However, the true positive group had less mature eggs and poorer fertilization rate; this may be due to absence of hCG. It is therefore advisable to administer hCG regardless of spontaneous LH surge.

5. It was not necessary to do repeated ultrasound scans if daily  $E_2$  results were available; it was also possible to predict when the spontaneous LH was likely to occur based on the  $E_2$  levels and the number of follicles.
6. When the luteal phase was supported with daily intramuscular P, there were significantly higher plasma levels of P on days 10 and 12, seen when there was spontaneous LH. As expected the placebo group had a shorter luteal duration (13.0 days, compared to 14.8 days in the P-supported group); however, there was no markedly low P levels in the placebo group.

I wish to emphasize that all the endocrine manipulation for overstimulation in IVF cycles can result in abnormalities not conducive to the final outcome, i.e. to obtain a pregnancy. Centres all over the world are trying to find a balance between stimulation and optimal results.

We are far from this balance at the moment. Yuen, Pride, Rowe et al (1985) reported on a controlled trial with a low-dose "nonsuperovulation" and a high-dose, individually adjusted "controlled-superovulation" regimen with hMG in women with normal cycles. In the latter regimen, more hMG was used (19.8 versus 10.3 ampoules for the "controlled-superovulation" regimen and "nonsuperovulation" regime respectively),  $E_2$  levels were higher (1288 pg/ml versus 343 pg/ml), more eggs were retrieved (53 oocytes from 18 cycles versus 25 oocytes from 11 cycles), and more eggs were mature (87% versus 44%). Moreover, there were more eggs that fertilized and cleaved (72% versus 52%), with 2 pregnancies in 18 cycles in the "controlled-superovulation" regime (11%) while there were no pregnancies in 11 cycles in the "nonsuperovulation" regime. The authors concluded that the higher-dose "controlled-superovulation" regime was better for IVF.

However, as we understand more of the negative or detrimental effects of superovulation, there may be a swing towards the other extreme.



".....now that the optimal conditions for oocyte retrieval and fertilisation in vitro and for embryo culture and transfer are more clearly defined and standardised, it would be worth considering a return to the use of natural unstimulated cycles. Although they only yield a single oocyte they do so within a more physiological endocrine environment."

Jeffcoate SL (1985)

## CHAPTER 12

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